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PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR

TESE DE DOUTORADO

Efeitos de estrógenos ambientais sobre a reprodução do lambari *Astyanax rivularis* no Alto Rio das Velhas, bacia do Rio São Francisco.

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Belo Horizonte

2018

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Tese apresentada ao Programa de Pós-graduação em Biologia Celular da Universidade Federal de Minas Gerais com requisito para a obtenção de título de Doutor em Ciências (Área de concentração em Biologia Celular).

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ATA DA DEFESA DE TESE DE DOUTORADO DE

ANDRÉ ALBERTO WEBER

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Às **nove horas** do dia **19 de fevereiro de 2018**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora da Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho final intitulado: **"EFEITOS DE ESTRÓGENOS AMBIENTAIS SOBRE A REPRODUÇÃO DO LAMBARI ASTYANAX RIVULARIS NO ALTO RIO DAS VELHAS, BACIA DO RIO SÃO FRANCISCO "**, requisito final para obtenção do grau de Doutor em Biologia Celular. Abrindo a sessão, a Presidente da Comissão, **Dra. Elizete Rizzo**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra ao candidato, para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa do candidato. Logo após, a Comissão se reuniu, sem a presença do candidato e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	Instituição	Indicação
Dra. Elizete Rizzo	UFMG	APROVADO
Dra. Cleida Aparecida de Oliveira	UFMG	APROVADO
Dra. Gleide Fernandes de Avelar	UFMG	APROVADO
Dr. Hélio Batista dos Santos	UFSJ	APROVADO
Dr. José Enemir dos Santos	PUC MINAS	APROVADO

Pelas indicações, o candidato foi considerado: APROVADO

O resultado final foi comunicado publicamente ao candidato pela Presidente da Comissão. Nada mais havendo a tratar, a Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora. **Belo Horizonte, 19 de fevereiro de 2018.**

Dra. Elizete Rizzo (Orientadora) ____________________

Dra. Cleida Aparecida de Oliveira ____________________

Dra. Gleide Fernandes de Avelar ____________________

Dr. Hélio Batista dos Santos ____________________

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Profa. Luciana de Oliveira Andrade
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O presente trabalho foi desenvolvido no laboratório de Ictiohistologia do Departamento de Morfologia, Instituto de Ciências Biológicas, UFMG, com apoio técnico do Laboratório de Cromatografia do Departamento de Química da UFMG e do Centro de Microscopia da Universidade Federal de Minas Gerais (CM-UFMG).

Orientadora
Dra. Elizete Rizzo

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“*You got to lose to know how to win…..Dream on, dream on, dream on*
And dream until your dreams come true”
Aerosmith

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Resumo

O rio das Velhas, importante afluente da bacia do rio São Francisco, é considerado o rio mais poluído do estado de Minas Gerais, devido à alta carga de contaminação aquática proveniente de esgoto doméstico e industrial. Os principais objetivos desse estudo foram avaliar os efeitos de estrógenos ambientais sobre a reprodução da espécie *Astyanax rivularis*, espécie de pequeno porte encontrada em riachos com altitudes elevadas no alto rio das Velhas e avaliar a imunomarcação de CYP19, ERα e ERβ nos testículos ao longo do desenvolvimento gonadal e entre sites com diferentes níveis de contaminação estrogênica. Foram realizadas quatro coletas de campo em três córregos: S1 (baixa interferência antrópica) S2 e S3 (submetidos a contaminação por esgoto doméstico sem tratamento). Foram avaliados, em machos e fêmeas dessa espécie, a expressão hepática de vários biomarcadores moleculares (*Zrp*, *Vtg*, *IGF-I, CYP19, ERα e ERβ*), histológicos (histopatologia de ovócitos e intersexo), 'endpoints' reprodutivos (índices gonadossomático, hepatossomático, fator de condição de Fulton, fecundidade e proporção de células da linhagem gametogênica) e desvios na proporção sexual nos diferentes sites de coleta. Foram avaliados os parâmetros físico-químicos por sonda Horiba e os compostos estrogênicos através de cromatografia líquida acoplada a espectrometria de massas (LC-MS). Os sites expostos a esgoto doméstico apresentaram níveis elevados de estradiol, estriol, estrona, bisphenol A e nonilfenol quando comparado com S1. Os parâmetros físico-químicos (temperatura e oxigênio dissolvido) não tiveram diferenças entre os sites de coleta. Os resultados indicam desregulação endócrina nos sites expostos a esgotos domésticos (S2 e S3), com aumento na proporção de fêmeas, aumento dos níveis de *Vtg*, *Zrp*, *CYP19*, *ERα* e *ERβ* em machos e diminuição de *Vtg* em fêmeas. O *IGF-I* de machos em S2 apresentou-se baixo em relação ao site referência. Em S2 e S3 foi observado proporção alta de ovócitos deficientes em vitelo e em S3 houve aumento de ovócitos supermaturados. A proporção de atresia folicular foi baixa e não apresentou diferenças significativas entre as populações de fêmeas amostradas. A condição de intersexo foi encontrada apenas nos sites expostos a esgoto doméstico. Análises morfométricas da gametogênese demonstraram maior proporção de ovócitos vitelogênicos e espermatozoides em S1 do que os peixes capturados nos sites S2 e S3. Os resultados da imunohistoquímica demonstraram marcação de CYP19 em células de Leydig e granulócitos acidófilos, espermatogônia, células de Sertoli, espermátides e espermatozóides. ERα apresentou

distribuição mais ampla do que ERβ sendo encontrado em todas as fases de desenvolvimento de células germinativas. Por outro lado, ERβ foi encontrado apenas em espermatogônia e espermatócitos. Ambos ERs foram expressos nas células Leydig e Sertoli. Durante a maturação testicular, os níveis de ELISA para CYP19, ERα e ERβ seguiram o índice gonadossomático (GSI) com valores significativamente maiores no estágio maduro. Os efeitos reprodutivos observados estão relacionados com o aumento da urbanização e liberação de esgoto doméstico diretamente nos corpos d'água sem nenhum tratamento e mesmo que as populações de *A. rivularis* estejam habitando ambientes lóticos e com alta renovação de água a liberação de contaminantes por esgoto doméstico é constante, promovendo assim uma exposição crônica de peixes a esses desreguladores endócrinos.

Palavras-chave: estrógenos ambientais, receptores nucleares, desreguladores endócrinos, histopatologia gonadal, teleósteos.

Abstract

The Velhas River, an important tributary of the São Francisco river basin, is considered the most polluted river in the state of Minas Gerais, due to the high amount of water contamination from domestic and industrial sewage. The main objectives of this study were to evaluate the effects of environmental estrogens on the reproduction of the species *Astyanax rivularis*, a small species found in streams with high altitudes in the upper Rio de Velhas and to evaluate the immunostaining of CYP19, ERα and ERβ in the testis along the gonadal development and among sites with different estrogenic contamination. Four field samples were collected in three streams: S1 (low anthropogenic interference) S2 and S3 (contaminated by domestic sewage without treatment). The hepatica expression of a number of molecular (*Zrp, Vtg, IGF-I, CYP19, ERα* and *ERβ*) and histological biomarkers (oocytes histopathology and intersex), reproductive endpoints (gonadossomatic, hepatossomatic, Fulton, fecundity and proportion of gametogenic lineage cells) and deviations in the sexual proportion were assessed in the different collection sites. The physicochemical parameters were evaluated by Horiba probe and estrogenic compounds by liquid chromatography coupled to mass spectrometry (LC-MS). Sites exposed to domestic sewage presented high levels of estradiol, estriol, estrone, bisphenol A and nonylphenol when compared to S1. The physical-chemical parameters (temperature and dissolved oxygen) had no differences between the collection sites. The results indicate endocrine disruption in sites exposed to domestic sewage (S2 and S3), such as increase in the proportion of females, increase of *Vtg, Zrp, CYP19, ERα* and *ERβ* levels in males and decrease of *Vtg* in females. Male *IGF-I* in S2 was low in relation to the reference site. In S2 and S3, a high proportion of yolk deficient oocytes was observed, and in S3 there was an increase in over-ripening oocytes. The proportion of follicular atresia was low and did not present significant differences among the populations of females sampled. The intersex condition was found only in sites exposed to domestic sewage. Morphometric analyzes of gametogenesis demonstrated a higher proportion of vitellogenic oocytes and spermatozoa in S1 than fish captured at sites S2 and S3. The results of immunohistochemistry demonstrated CYP19 labeling in Leydig cells and acidophilic granulocytes, spermatogonia, Sertoli cells, spermatids and spermatozoa. ERα showed a broader distribution than ERβ being found in all stages of germ cell development. On the other hand, ERβ was found only in spermatogonia and spermatocytes. Both ERs

were expressed in Leydig and Sertoli cells. During testicular maturation, ELISA levels for CYP19, ERα and ERβ followed the gonadosomatic index (GSI) with significantly higher values at the mature stage. The observed reproductive effects are related to the increase of urbanization and release of domestic sewage directly into water bodies without any treatment and even if the populations of *A. rivularis* are inhabiting lotic environments and with high water renewal the release of pollutants through sewage is constant, thus promoting chronic exposure of fish to these endocrine disrupters.

Keywords: environmental estrogens, nuclear estrogen receptors, endocrine disrupters, gonadal histopathology, teleosts.

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1. Introdução Geral

1.1. Contaminação aquática e desreguladores endócrinos

A poluição crescente de rios, lagos e córregos de água doce têm provocado sérias consequências para a biota aquática. De acordo com a Sociedade Americana de Químicos foram encontradas mais de 10 milhões de substâncias exógenas nos corpos d'água e, desse total, aproximadamente 2 mil causam efeitos negativos sobre a fauna aquática, principalmente em peixes (Venâncio and Domingos, 2014). No entanto, não se sabe os efeitos que esses químicos promovem sobre a fisiologia de espécies aquáticas. Recentemente, resíduos de fármacos antidepressivos e estatinas (que atuam na redução de colesterol) foram encontrados em tecidos de peixes de água doce (Grabicova et al., 2014; Santos et al., 2016).

Substâncias exógenas que interagem com sistema endócrino de animais e humanos recebem o nome de desreguladores endócrinos (EDC's). Essas substâncias podem alterar a homeostase celular mediante receptores específicos ou independente de receptores (Figura 1). As vias dependentes de receptores podem afetar a transdução de sinais–através de receptores de membrana (ex. receptores de estrógeno acoplado a proteína G) ou intracelulares (ex. receptores de estrógeno α e β), dessa forma causando alterações na transcrição gênica (Babin et al., 2007).

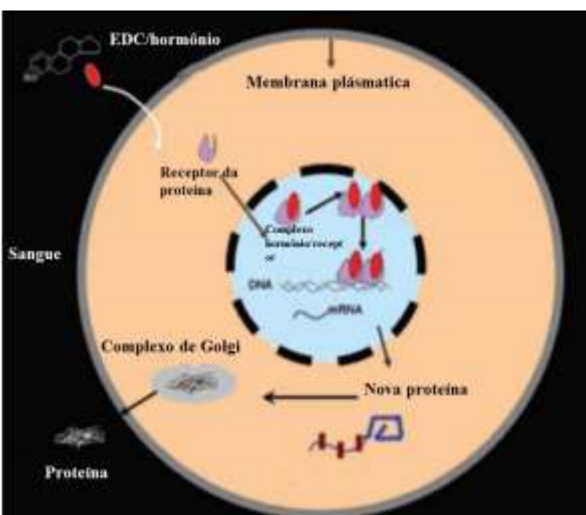

Figura 1 - Atuação de desreguladores endócrinos (EDC) atuando via receptor. Reproduzido de Denslow and Sepúlveda, 2007.

Os desreguladores endócrinos podem atuar na via esteroidogênica como agonista e/ou antagonista de estrógenos. Os antagonistas podem se ligar a um receptor específico e bloquear totalmente a resposta da célula ou torna-la insuficiente. Por outro lado, os agonistas mimetizam a atuação do ligante, aumentando a resposta da célula (Figura 2) (Babin et al., 2007). Geralmente, estudos toxicológicos têm como objetivo entender os efeitos de contaminantes atuando isoladamente nos organismos aquáticos. No entanto, a interação de diversos compostos pode gerar respostas ou efeitos totalmente distintos das observadas em contaminante atuando isoladamente (Luzio et al., 2015). Estrógenos ambientais podem causar vários efeitos negativos sobre a reprodução humana, como declínio do esperma, feminilização em homens e indução de menopausa precoce e virilização em mulheres (Bolong et al., 2009; Sumpter and Jobling, 2013).

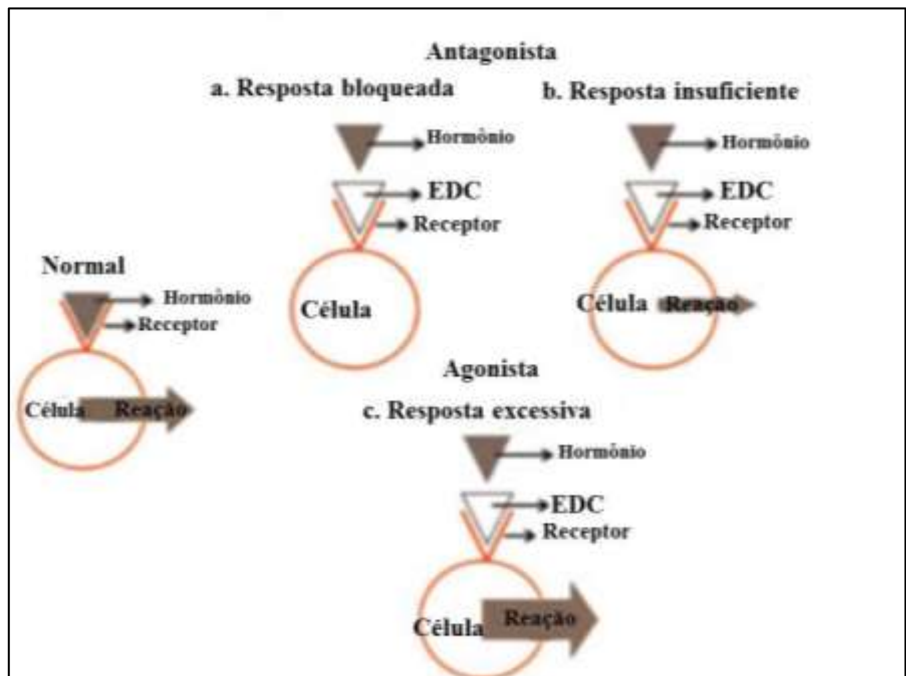

Figura 2. Mecanismos de ação de desreguladores endócrinos (EDC), atuando em receptores de hormônios. EDC podem atuar como antagonistas e impedir ou reduzir a resposta (a) e (b); ou podem agir como agonistas e aumentar a resposta (c). Adaptado de Denslow and Sepúlveda, 2007.

1.2. Regulação endócrina da reprodução de peixes e atuação de EDC's estrogênicos

A dinâmica reprodutiva de peixes é regulada pelo eixo hipotálamo-hipófise-gônada (HHG), através das gonadotrofinas (Redding and Patino, 1993). Condições ambientais como temperatura e fotoperíodo estimulam a secreção do hormônio liberador de gonadotrofinas (GnRH) pelo hipotálamo (Redding and Patino, 1993). O

GnRH estimula a produção pela hipófise de gonadotrofinas, GtH I e GtH II, que são similares funcionalmente aos hormônios folículo estimulante (FSH) e hormônio luteinizante (LH), respectivamente. Em fêmeas, o FSH está envolvido com a vitelogênese e zonagênese, enquanto que o LH atua na maturação final ovocitária e ovulação (Lubzens et al., 2010). Em machos, o FSH regula o funcionamento da célula de Sertoli, como nutrição e regulação do desenvolvimento das células germinativas e o LH atua regulando as células de Leydig na produção de esteroides sexuais (Huhtaniemi and Themmen, 2005).

Em peixes, os principais esteroides sexuais são a testosterona (T) e a 11-ketotestosterona (11-KT) nos machos (Schulz et al., 2010) e em fêmeas, o 17β-estradiol (E2) que é produzido através da aromatização da testosterona pela enzima P450 aromatase (CYP19) (Lubzens et al., 2010). Mesmo que E2 seja considerado hormônio feminino, também é encontrado em menor concentração nos machos, sendo importante na manutenção da fertilidade (Robertson et al., 2001). A produção de E2 pelos ovários estimula o fígado a sintetizar proteínas que serão utilizadas na maturação dos ovócitos, vitelogenina (Vtg), assim como as proteínas da zona radiata (Zrp) que formam o envelope vitelínico (Modig et al., 2007). Em peixes, esse envelope é formado por três proteínas (Zrpα, Zrpβ e Zrpγ) (Lubzens et al., 2010), sendo essa etapa precedente a chegada de precursores do vitelo ao ovócito. A Vtg é incorporada pelo ovócito através da passagem pelas células da teca e foliculares até atingirem os poros canais da zona radiata. Nas células foliculares, esse processo ocorre por endocitose de vesículas, envolvendo receptores específicos mediados por clatrina (Wallace and Selman, 1990). Nos ovócitos, as vesículas contendo Vtg se fundem a lisossomos formando assim corpos multivesiculares. Dentro desses corpos, a Vtg é clivada por enzimas lisossomais, como a Catepsina-D, em proteínas menores, lipovitelina e fosvitina, para formar os glóbulos de vitelo (Carnevali et al., 1999).

Além do eixo HHG, estudos demonstram que o eixo somatotrófico constituído por hormônio do crescimento-fator de crescimento semelhante a insulina (GH-IGF) interage com o eixo HHG e atua na modulação da esteroidogênese testicular e ovariana (Hull and Harvey, 2014). A liberação de hormônio do crescimento (GH) pela hipófise estimula a produção de fatores de crescimento semelhante à insulina (IGF-I e IGF-II) pelo fígado que são importantes no crescimento e reprodução de peixes (Pierce et al., 2005). Na espécie *Oncorhynchus tshawytscha,* a elevação de IGF-I promove o início da proliferação espermatogonial em indivíduos jovens (Campbell, 2003). Em fêmeas, o

GH potencializa os efeitos de E2 estimulando assim a síntese de vitelogenina (Vtg) e o IGF-I promove um aumento na expressão de CYP19 (Kagawa et al., 2003; Nakamura et al., 2003).

Os EDC's estrogênicos são aqueles capazes de mimetizar a atuação do estrógeno natural (E2) e dessa forma podem alterar a síntese de proteínas pelo fígado. Os EDC's estrogênicos são divididos em dois grupos: naturais e sintéticos. Esses compostos atingem o ambiente aquático principalmente através da liberação de esgoto doméstico, escoamento agrícola e esgoto industrial (Atkinson, 2008). Estrógenos naturais que são encontrados em descargas de esgoto doméstico incluem estrona (E1), 17β-estradiol (E2) e estriol (E3). Seres humanos excretam distintas quantidades de esteroides, dependendo da idade, estado de saúde, dieta ou gravidez. Homens eliminam diariamente através da urina aproximadamente 3 μg de cada estrógeno natural, enquanto que as mulheres podem eliminar entre 0.6 a 9850 μg diariamente (Tabela 1). Os estrógenos naturais atingem os sistemas aquáticos através da liberação de esgoto doméstico e são oriundos do metabolismo humano, animal e vegetal. Por outro lado, os estrógenos sintéticos (xenoestrógenos) apresentam várias origens e derivam de atividades agrícolas, plásticos industriais e produtos farmacêuticos e atingem corpos d'água através da lixiviação e escoamento (Figura 3). Dentre os estrógenos sintéticos amplamente encontrados no ambiente aquático destacam-se os compostos alquifenólicos (eg. nonilfenol), presentes em pesticidas e da degradação de detergentes não iônicos e o bisfenol A, monômero utilizado na fabricação de plásticos e resinas (Lintelmann et al., 2003). Esses compostos possuem variados graus de estrogenicidade, dependendo da sua afinidade com os receptores de estrógeno (de Voogt and van Hattum, 2003).

Tabela 1. Excreção humana diária de estrógenos naturais em μg/dia por pessoa.

	E1	**E2**	**E3**
Mulher grávida	787	277	9850
Mulher menopausa	31.5	59.2	90.7
Mulher menstruação	9.32	6.14	17.4
Mulher	7	2.4	4.4
Homem	3.5	1.83	3.21
Menino	0.63	0.54	-
Menina	0.6	2.5	0.918

Adaptado de Adeel et al., 2016.

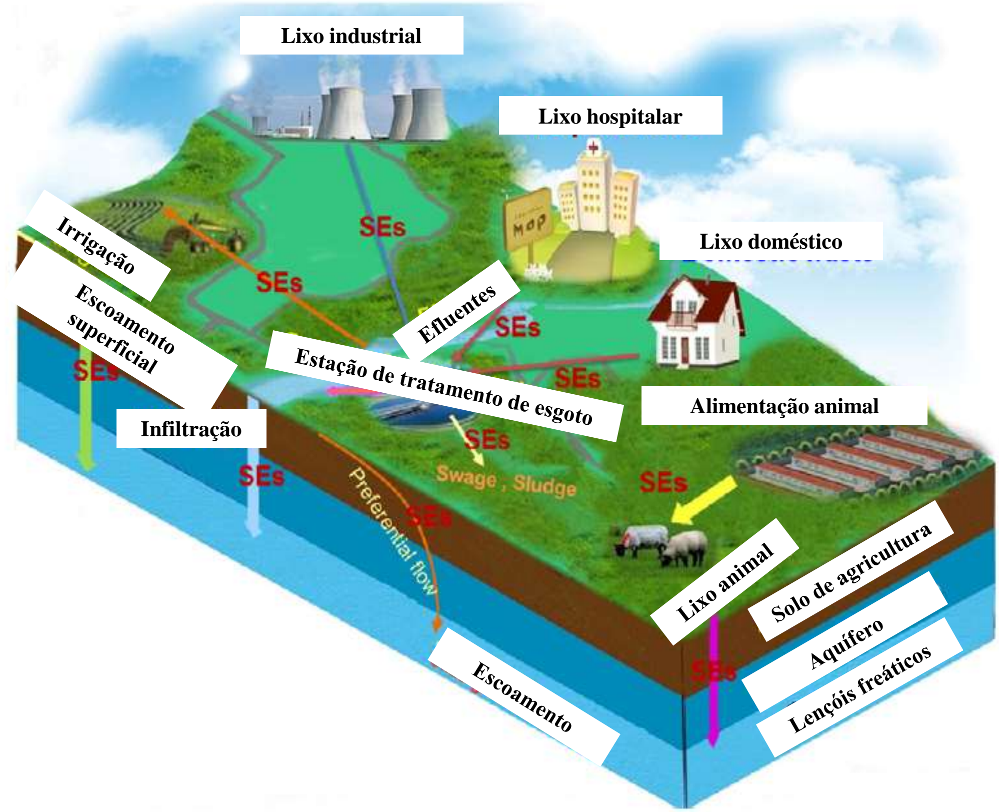

Figura 3. Principais fontes de exposição a estrógenos naturais e xenoestrógenos. Adaptado de Adeel et al., 2016.

1.3. Biomarcadores reprodutivos de desregulação endócrina

Os biomarcadores de desregulação endócrina podem ser identificados de acordo com o estágio de desenvolvimento das gônadas. Peixes expostos a EDC’s em estádio de maturação pode levar a alteração no tamanho gonadal e desregular as concentrações de esteroides sexuais (Bahamonde et al., 2013). Por outro lado, peixes expostos a EDC’s entre os estágios pós-desova e maturação gonadal podem desenvolver gônadas intersexo (Figura 4). Biomarcadores reprodutivos podem ser identificados em diferentes níveis da organização biológica, desde molecular até populacional. Biomarcadores moleculares e celulares possuem alta relevância mecanística, enquanto biomarcadores de alta relevância ecológica identificam os efeitos a níveis individuais e populacionais (Denslow and Sepúlveda, 2007).

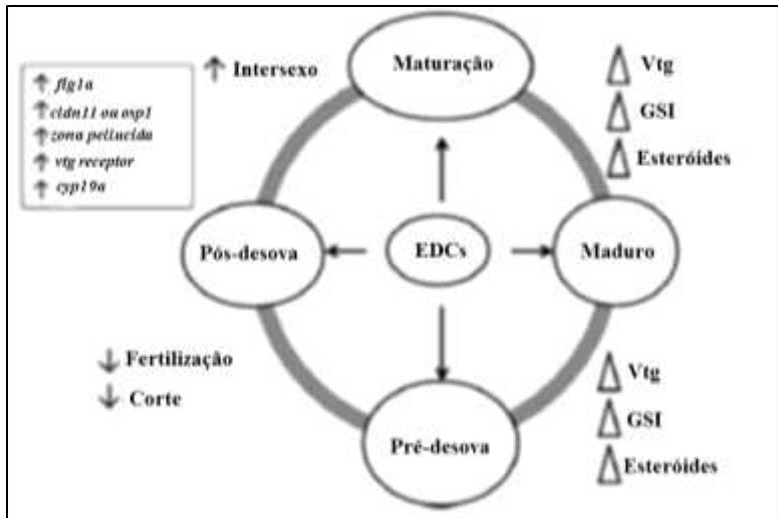

Figura 4. Diferentes biomarcadores de desregulação endócrina de acordo com o desenvolvimento da gônada. Adaptado de Bahamonde et al., 2013.

Alterações no crescimento gonadal são utilizadas como indicador de mudanças nos eixos HHG e GH-IGF. A crônica exposição a xenoestrógenos como nonilfenol, bisfenol A e 17α-etinilestradiol (EE2) pode inibir o crescimento testicular e ovariano em peixes adultos, alterando o índice gonadossomático (Rasmussen and Korsgaard, 2004; Silva et al., 2012; Sohoni et al., 2001).

Proteínas como Vtg e Zrp (Adeogun et al., 2016; Prado et al., 2014, 2011), IGF-I (Prado et al., 2014; Reinecke, 2010) e citocromo P450 (CYP1A) (Madureira et al., 2012) são considerados biomarcadores de relevância mecanística. A Vtg é o biomarcador mais utilizado em estudos de desregulação endócrina. A transcrição da Vtg é fortemente regulada por 17β-estradiol via receptores de estrógeno (ERs). Em peixes, são encontrados vários isotipos de receptores de estrógeno (ERα, β e γ) e a expressão destes receptores têm sido investigadas em poucas espécies de peixes, principalmente em condições de contaminação por EDCs (Kloas et al., 2000; Lange et al., 2008). Em trechos contaminados por compostos estrogênicos do rio Pearl na China foi encontrada uma elevada expressão de ERα em machos de *Gambusia affinis* (Wen et al., 2013).

Algumas histopatologias e alterações nas células germinativas também são observadas em peixes expostos a EDCs estrogênicos. Adultos de *Danio rerio* expostos a EE2 apresentaram alterações patológicas como fibrose intersticial e líquido proteináceo provavelmente derivado da degradação da Vtg e sua presença em ovários e testículos geralmente tem sido associada com a exposição a substâncias estrogênicas (OECD, 2009; Silva et al., 2012). Outras alterações como alargamento dos túbulos seminíferos, infiltração de adipócitos, desenvolvimento assincrônico do testículo e destacamento da

membrana basal são outras histopatologias observadas em peixes expostos a contaminantes estrogênicos (Luzio et al., 2016).

Diferenciação gonadal, fecundidade e alterações na proporção sexual são biomarcadores analisados em estudos envolvendo desregulação endócrina a nível populacional. A exposição a estrógenos ou andrógenos durante períodos críticos do desenvolvimento dos peixes assim como diferenciação sexual pode resultar em anormalidades sexuais irreversíveis e promover acentuados desvios na proporção sexual (Figura 5). Quando a exposição à esses EDC's ocorre na fase adulta é possível observar indivíduos intersexo que são caracterizados pela presença de tecido testicular e ovariano na mesma gônada (Jobling et al., 2002). Esse fenótipo foi observado em 37 espécies de peixes de água doce em 24 países e tem sido associado com a exposição a efluentes domésticos municipais (Bahamonde et al., 2013). Estudos recentes relatam que o intersexo pode ser classificado de acordo com sua severidade. Dependendo da concentração dos desreguladores estrogênicos nos corpos d'água pode-se observar desde poucos ovócitos perinucleolares (baixa severidade) até vários ovócitos, incluindo ovócitos vitelogênicos (alta severidade) ocupando grande parte do tecido testicular (Bahamonde et al., 2015).

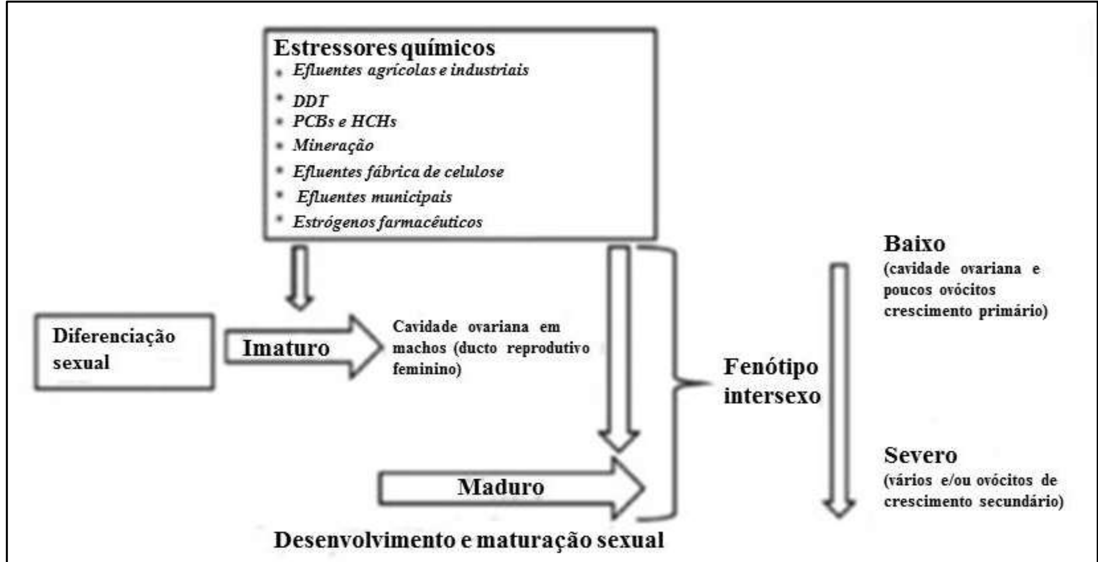

Figura 5. Períodos críticos de exposição aos EDC's nas diferentes fases do desenvolvimento. Adaptado de Bahamonde et al., 2013.

1.4. Aromatase (CYP19) e receptores de estrógeno (ERα e ERβ)

Os esteroides sexuais incluem os estrógenos, andrógenos e progesteronas os quais são derivados do colesterol. Na sua biossíntese, testosterona é diretamente derivado da androstenediona, enquanto que estrógenos são derivados da aromatização da testosterona (T) em 17β-estradiol (E2) ou androstenediona em estrona (E1) (Nelson and Habibi, 2013). Os peixes codificam duas enzimas aromatase, as quais são responsáveis pela aromatização de andrógenos em estrógenos, CYP19a encontrada nas gônadas e CYP19b encontrada no cérebro (Callard et al., 2001) (Figura 6).

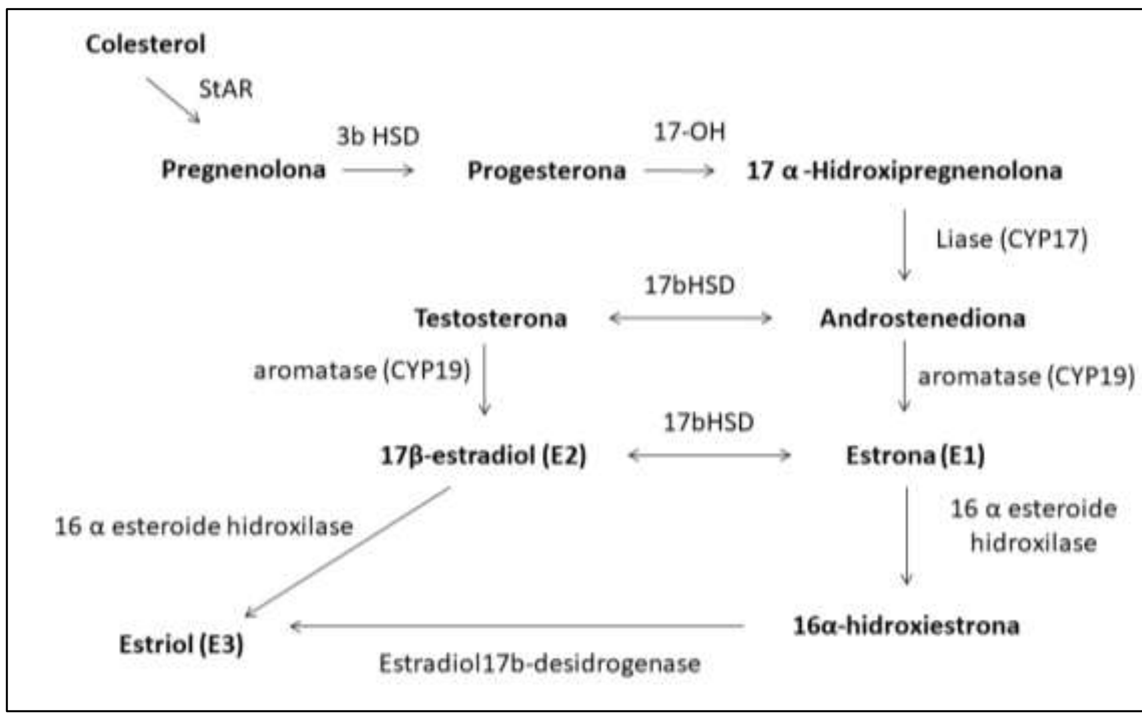

Figura 6 – Via de síntese do estrógeno em peixes teleósteos. Principais enzimas que participam da biossíntese do estrógeno. Reproduzido de Burgos-Aceves et al., 2016.

Estrógenos estão envolvidos em vários processos reprodutivos como regulação da ovogênese, vitelogênese, desenvolvimento testicular e também possui várias funções regulatórias em vários órgãos como os do sistema imune (Gustafsson, 2003; Hess, 2003). Estrógenos são conhecidos como moduladores das respostas imunes em peixes teleósteos (Harris and Bird, 2000). Essa interface entre sistemas imune-endócrino é parcialmente mediada pelas interações entre os estrógenos circulantes e os ERs encontrados nos leucócitos (Burgos-Aceves et al., 2016). Os estrógenos se ligam e ativam receptores de estrógeno citoplasmáticos (ER’s). Três ERs foram identificados em peixes ERα, ERβ e um não reconhecido ERγ (Nelson and Habibi, 2013). Os ER’s, assim como a maioria dos receptores nucleares, contêm seis regiões. A região N-

terminal (A/B) é a mais variável entre as espécies e contém a função de primeira ativação (AF1) (Weigel, 1996). Essa região pode ser fosforilada pela MAP quinase (MAPK) em resíduos de serina e treonina (Kato et al., 1995). O domínio C dos ER's é também conhecido como ligante do DNA (DBD). Essa região é altamente conservada entre as espécies de vertebrados. Essa região contém dois dedos de zinco (zinc fingers) que permitem a interação desses receptores com sequências específicas do DNA, denominadas elementos responsivos a estrógeno (EREs) (Schwabe et al., 1995). A região D é fracamente conservada evolutivamente, sendo uma região que confere sinais de localização nuclear. Algumas evidências sugerem que co-repressores dos ERs podem interagir nessa região (Aranda and Pascual, 2001). A região E, na qual é encontrado o domínio de ligação do ligante (LBD), é responsável por induzir a função de segunda ativação (AF2) e interação com estrógeno (Aranda and Pascual, 2001). Além disso, essa região media a dimerização e interação com as HSP's e proteínas co-reguladoras (Folkertsma et al., 2004). A região F, encontrada na porção C-terminal dos ER's determina o fim do AF2, sendo que ainda não foram descritas funções para essa região (Nelson and Habibi, 2013) (Figura 7).

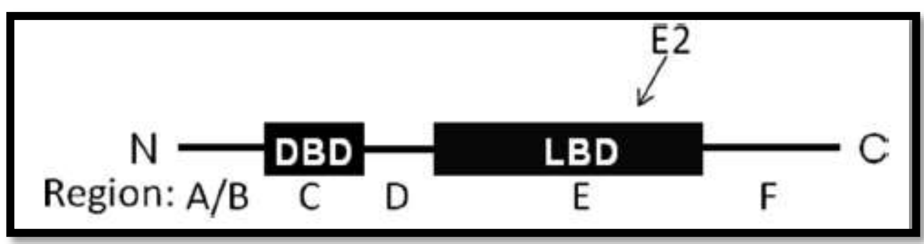

Figura 7 – Estrutura dos receptores de estrógeno e suas regiões específicas. Reproduzido de Nelson & Habibi, 2013.

Na literatura são descritos dois processos de atuação celular dos estrógenos, a via clássica (genômica) e a via não clássica (não genômica) (Nelson and Habibi, 2013). A via gênomica de atuação dos estrógenos envolve a ligação do estrógeno aos seus receptores (ERs) presentes no citoplasma (O'Malley and Tsai, 1992). Na ausência do ligante (estrógeno), esses ER's formam complexos com proteínas do choque térmico (HSP's) inibitórias (Nelson and Habibi, 2013). Na presença do estrógeno, as HSP's são removidas e esses receptores se movem para o núcleo, em regiões específicas do DNA, chamadas de ERE's (elementos responsivos a estrógeno), resultando em alterações nas taxas de transcrição de genes. Essa via é relativamente lenta e ocorre em uma escala de

horas (Thomas, 2012) (Figura 8). Em peixes teleósteos, o ERβ possui maior afinidade pelo estradiol, atuando assim como um sensor. Dessa forma, assim que o estradiol circulante aumenta no corpo do animal ocorre também um aumento nos níveis de ERα, assim desencadeando a vitelogênese (Figura 9). Devido à completa duplicação do genoma durante a evolução, diversas espécies de peixes podem ter mais do que uma isoforma de ERα, ERβ e P450 aromatase (Nelson and Habibi, 2013).

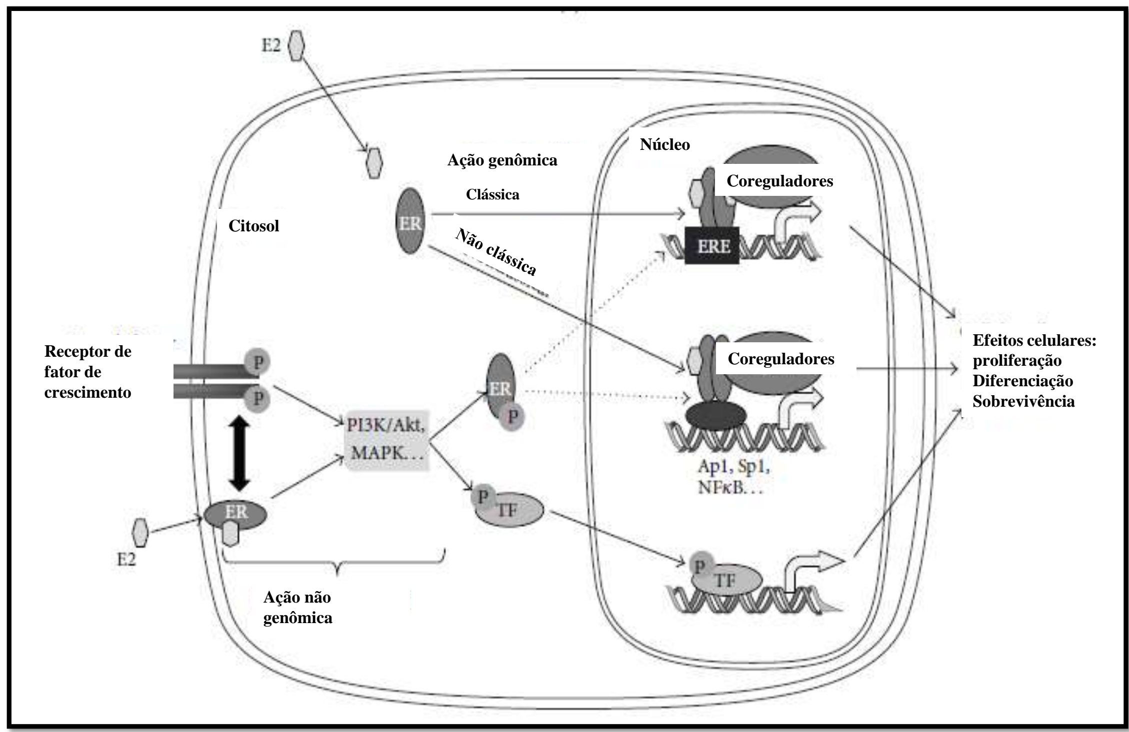

Figura 8 – Mecanismo de ação dos receptores de estrógeno. Adaptado de Kerdivel et al., 2013.

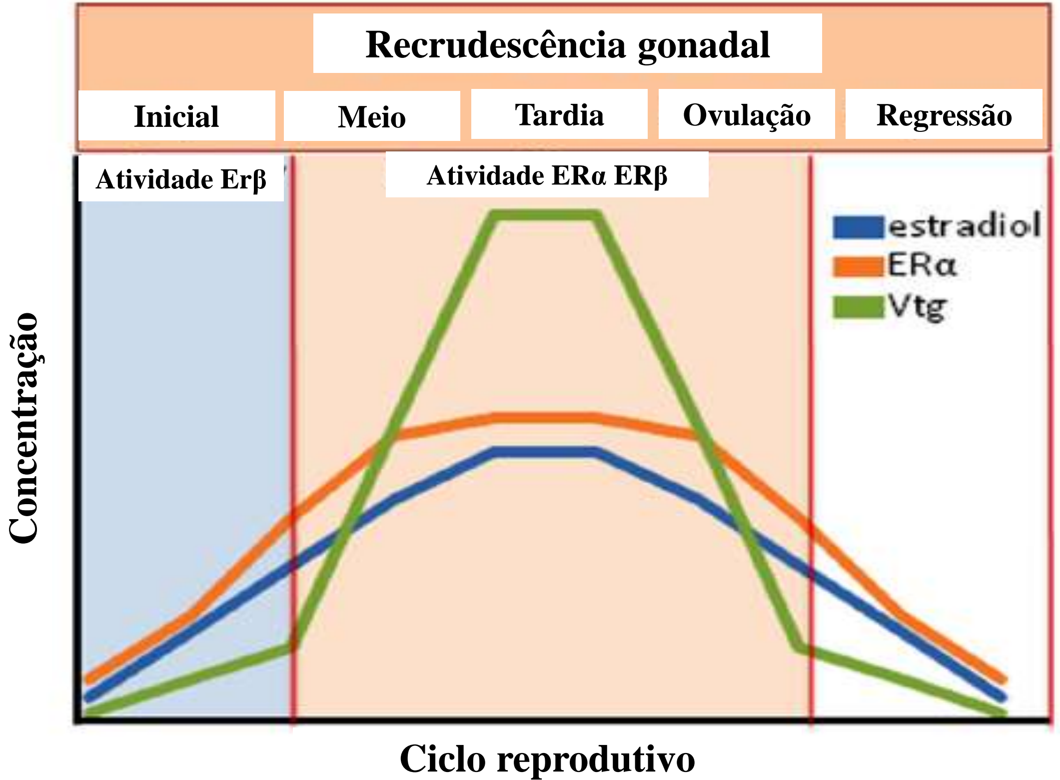

Figura 9 – Modelo de auto-regulação e função de ER's em peixes. Reproduzido de Nelson & Habibi, 2013.

Além da via genômica foi descrito por Szego e Davis, 1967 uma via rápida de atuação do estrógeno, denominada não genômica que envolve receptores de membrana. A proteína de membrana associada com a atuação do estrógeno é a GPR30 (Receptor 30 associado a proteína G) e foi descrita a primeira vez por Filardo e colaboladores em 2002. Estudos com células cancerígenas, SKBR3, que não expressam ERα e ERβ, submetidas a ação de estrógenos demonstravam rápida ativação de mensageiros secundários como cAMP e ERK (Filardo, 2002). Recentemente, foi descrito uma regulação entre mPRs (via não gênomica de progestinas) e GPR30 (via não genômica de estrógenos) por estrógenos e dihidroxiprogesterona (DHP) em ovócitos de zebrafish regulando o início da maturação ovocitária (Figura 10).

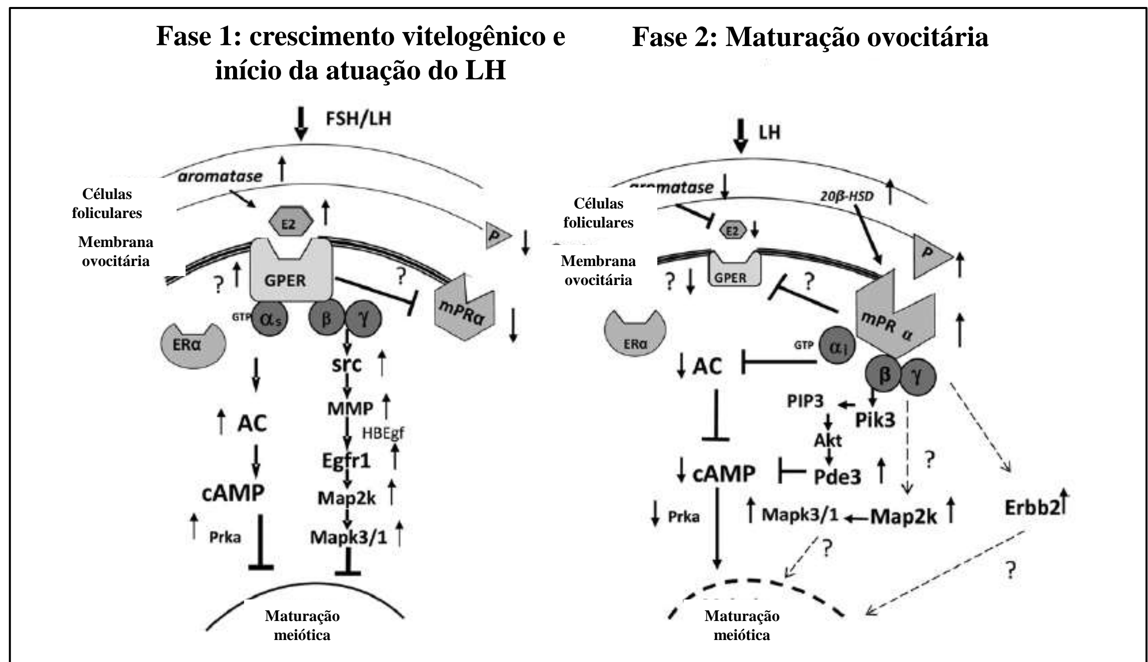

Figura 10 - Modelo proposto do controle duplo do início da maturação de ovócitos em teleósteos por estrógenos e progestinas que atuam através de GPR30 e mPRa, respectivamente, em diferentes estágios do desenvolvimento de ovócitos. Reproduzido de Thomas, 2012.

1.5. Bioindicadores de desregulação endócrina

Diferentes espécies de peixes têm sido utilizadas como sentinelas em estudos ecotoxicológicos de campo ou de laboratório tais como *Rutilus rutilus* (Bjerregaard et al., 2006; Jobling et al., 2002), *Etheostoma caeruleum* (Bahamonde et al., 2015; Fuzzen et al., 2015), *Gambusia affinis* (Wen et al., 2013), *Danio rerio* (Luzio et al., 2016, 2015; Silva et al., 2012) e *Pimephales promelas* (Ankley et al., 2002). Em geral, espécies de pequeno porte, com ampla distribuição, alta abundância e curto ciclo reprodutivo são bioindicadores adequados para estudos de desregulação endócrina.

As espécies do gênero *Astyanax* apresentam ampla distribuição na região Neotropical e possuem alta abundância de indivíduos em corpos d'água com diferentes graus de contaminação. Por esses motivos, espécies desse gênero são consideradas apropriadas para estudos ecotoxicológicos na América do Sul (Paulino et al., 2014; Prado et al., 2014, 2011; Schulz and Martins-Junior, 2001; Tolussi et al., 2018).

1.6. Espécie de estudo

A espécie *Astyanax rivularis* (Lutken, 1875) (Figura 11) da família Characidae, gênero Incertae sedis, conhecida popularmente como lambari de riacho, possui pequeno porte corporal e habita riachos e córregos formados por corredeiras e substrato composto por rochas e pedras (Shibatta and Cheida, 2003). Possui distribuição em drenagens com altas altitudes da bacia do rio São Francisco. É uma espécie gonocorística e apresenta desova do tipo parcelada com desenvolvimento assincrônico dos ovócitos (Veloso-Júnior et al., 2009). Essa espécie alimenta-se de insetos aquáticos, larvas e plantas, sendo totalmente dependente de itens alóctones para sua alimentação. Essa espécie é abundante e possui ampla distribuição ao longo das drenagens de cabeceiras do rio das Velhas.

Figura 11. Exemplar de *Astyanax rivularis* (comprimento total médio 8 cm).

1.7. Área de estudo

O rio das Velhas está localizado na região central de Minas Geral sendo um dos principais tributários da bacia do rio São Francisco. Possui volume de água anual de 631m^3/s, com uma área correspondendo a 27.867 km^2 e extensão de 761 km (Alves and Pompeu, 2005). A cabeceira do rio das Velhas está localizado em uma área de transição entre vegetação de Mata Atlântica e Cerrado. Esses dois biomas são considerados “hotspots” de biodiversidade mundial, devido ao alto número de espécies endêmicas e alta perda de habitat (Myers et al., 2000).

O rio das Velhas é considerado o rio mais poluído do estado de Minas Gerais devido ao alto grau de ocupação humana de aproximadamente 5 milhões de pessoas ao longo dessa bacia. As principais fontes poluidoras dessa bacia são esgoto doméstico e industrial principalmente da região metropolitana de Belo Horizonte e atividades de mineração. Um fator agravante é a liberação de esgoto doméstico e industrial que é apenas parcialmente tratado (IBGE, 2014). Ao longo da calha principal do Rio das Velhas e em seus afluentes, como no Rio Itabirito, foram reportados recentemente a presença de vários compostos estrogênicos como 17α-etinilestradiol (EE2), estradiol

(E2), nonilfenol (NP) e bisphenol A (BPA) em elevadas concentrações (Moreira et al., 2011). Além disso, metais pesados como ferro, cromo, cobre, arsênio e ouro foram também encontrados na calha do Rio das Velhas e em seus afluentes de maior porte (Veado et al., 2000).

A área de estudo está localizada na porção alta dessa bacia próximo as cidades de Itabirito, Rio Acima e Nova Lima. O esgoto dessas cidades é despejado in natura dentro dos corpos d'água sem nenhum tratamento. Foram escolhidos três córregos, sendo um córrego considerado referência (S1) que não recebe esgoto de nenhuma fonte poluidora e dois que estão localizados dentro de centros urbanos, expostos a liberação de esgoto doméstico (S2 e S3) (Figura 12).

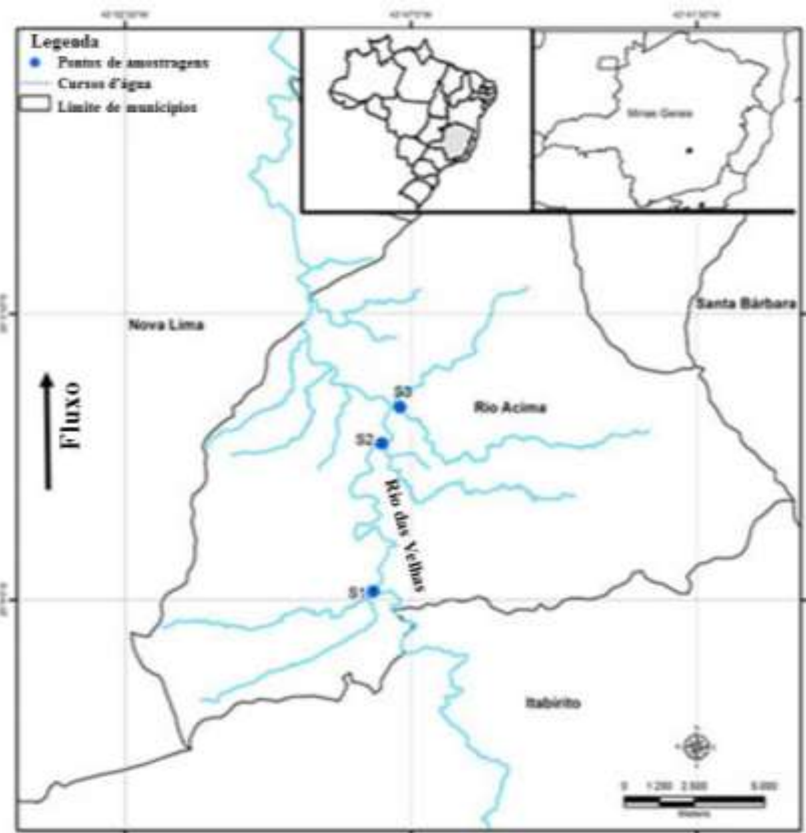

Figura 12. Localização geográfica da área de estudo e dos pontos de coleta no alto rio das Velhas.

2. Justificativa

Problemas com poluição do ambiente aquático são observados em diversas partes do mundo ocasionadas principalmente por descarga direta de efluentes domésticos e industriais que promovem um grande aumento de dejetos e outras substâncias químicas nos corpos d'água. Essa constante poluição sem tratamento adequado, afeta a fauna aquática de diferentes maneiras, incluindo contaminação por desreguladores endócrinos. No Brasil esse tema é ainda pouco explorado, mas de importância toxicológica relevante, tanto para o meio ambiente como para a saúde pública.

A carência de estudos dessa natureza em rios brasileiros demonstra a necessidade de um maior número de pesquisas nessa área de estudo. A maioria dos rios brasileiros apresenta contaminação por diferentes fontes poluidoras e o tratamento de esgoto na maioria dos casos é ineficiente. Dessa forma, a contaminação aquática é uma grande ameaça para a biota aquática incluindo peixes de água doce.

3. Objetivos

O objetivo do presente estudo foi analisar os efeitos de desreguladores endócrinos (EDCs) estrogênicos sobre a gametogênese e reprodução da espécie nativa *Astyanax rivularis* (Pisces: Characidae) no alto rio das Velhas, bacia do rio São Francisco.

Os seguintes objetivos específicos foram investigados:

- Avaliar os efeitos de EDCs estrogênicos sobre a espermatogênese e ovogênese de *A. rivularis*, utilizando biomarcadores populacionais (fecundidade, razão sexual, índices biológicos), individuais (intersexo), teciduais (histopatologias) e moleculares (Zrp, Vtg e IGF-I).

- Caracterizar a expressão estágio-específica de ERα, ERβ, CYP19 em testículos de *A. rivularis* capturados em ambiente com pouca atividade antrópica e, em adição, verificar se a exposição a EDCs estrogênicos interfere na expressão dessas proteínas em machos expostos a contaminação por esgoto doméstico.

4. Resultados

Os resultados obtidos no presente trabalho encontram-se sumarizados no itens abaixo:

4.1. Artigo 1 (Publicado na Science of The Total Environment)

WEBER, A.A.; MOREIRA, D.P.; MELO, R.M.C.; VIEIRA, A.B.C.; PRADO, P.S.; SILVA, M.A.N.; BAZZOLI, N.; RIZZO, E. (2017). Reproductive effects of oestrogenic endocrine disrupting chemicals in *Astyanax rivularis* inhabiting headwaters of the Velhas River, Brazil, 592: 693-703.

4.2. Artigo 2 (Submetido para publicação na Environmental Science and Pollution Research)

WEBER, A.A.; MOREIRA, D.P.; MELO, R.M.C.; VIEIRA, A.B.C.; BAZZOLI, N.; RIZZO, E. (2018). Stage-specific testicular protein levels of the oestrogen receptor (ERα and ERβ) and Cyp19 and association with oestrogenic contamination in the lambari *Astyanax rivularis* (Pisces: Characidae). Submitted.

4.1. Artigo 1

(Publicado na Science of The Total Environment)

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Reproductive effects of oestrogenic endocrine disrupting chemicals in *Astyanax rivularis* inhabiting headwaters of the Velhas River, Brazil

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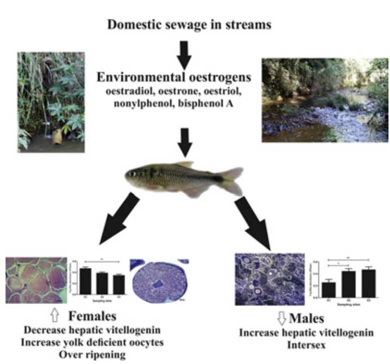
HIGHLIGHTS

- Oestrogenic EDCs were assessed by HPLC/MS in Velhas River headwaters, Brazil.
- Over-ripening and yolk deficient oocytes can be novel biomarkers of oestrogenic EDCs.
- Intersex gonads showed perinucleolar follicles into the seminiferous tubules.
- ELISA assays showed higher hepatic Vtg levels in males from impacted sites.
- Physiological follicular atresia occurred in sites contaminated by oestrogens.

GRAPHICAL ABSTRACT



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ABSTRACT

The Velhas River is the most polluted river in the state of Minas Gerais, south-eastern Brazil. Due to its historical and environmental relevance, the aim of this study was to evaluate the effects of oestrogenic endocrine disruptors on the reproduction of the lambari *Astyanax rivularis*, a small-sized species found in headwaters of the São Francisco River basin. Quarterly field samplings were carried out during a reproductive cycle in three streams of the upper Velhas River: S1 (reference site) and S2 and S3 (sites contaminated by untreated sewage). The main oestrogenic compounds were evaluated in water using HPLC/MS. Molecular, histological and reproductive biomarkers were assessed in liver and gonad. The results showed higher average concentrations of oestradiol (>200 ng/l) in S2 and S3, oestrone (>250 ng/l) in S2 as well as oestriol (>200 ng/l), bisphenol A (>190 ng/l), and nonylphenol (>600 ng/l) in S3 compared to S1 (<70 ng/l for all compounds). In S2 and S3, there was an increase in the proportion of females, higher ELISA levels of vitellogenin (Vtg) and proteins of the zona radiata (Zrp) in liver males. Insulin-like growth factor (IGF-I) levels were lower in S2 males, which also had a smaller body size, a smaller seminiferous tubule diameter, a higher proportion of spermatogonia, and lower proportion of spermatozoa in relation to S1. Histopathological analyses detected an increase in yolk deficient oocytes and over-ripening in the contaminated sites, and these alterations were associated to a reduction of hepatic Vtg levels and a delay in spawning, respectively. Intersex specimens with perinucleolar follicles in a multifocal distribution in the testis were detected in S2 and S3. These results indicate that chronic exposure to oestrogenic compounds induced endocrine disruption that may affect wild populations of *A. rivularis* in the Velhas River.

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1. Introduction

Untreated domestic sewage discharge into rivers and streams contains substances which have the ability to interact with the endocrine system of animals called endocrine disrupting chemicals (EDCs) (Babin et al., 2007). Among the EDCs, there are the oestrogenic endocrine disruptors, a subclass of compounds including both natural and synthetic oestrogens (Campbell, 2003). Natural oestrogens are mainly related to human metabolism, and population growth in areas adjacent to water bodies causes an increase of these substances in rivers, streams, lakes, and reservoirs. Synthetic substances from various sources such as agricultural, pharmaceutical, and industrial products that mimic the action of natural oestrogens (xenoestrogens) are also found in the aquatic environment (Barber et al., 2012).

EDCs can act in two critical windows of the development: sexual differentiation and gonadal maturation (Bahamonde et al., 2013). Exposure to EDCs in the early stages of development results in irreversible changes in tissue differentiation, which affect sex and in a long-term the reproductive potential of the offspring. In adults, endocrine disruption may be reversible, but chronic exposure to EDCs may compromise the viability of gametes and the sustainability of the species in its habitat (Denslow and Sepúlveda, 2007). Vitellogenin (Vtg) and zona radiata proteins (Zrp) are biomarkers widely used in endocrine disruption studies (Bahamonde et al., 2014; Kidd et al., 2007; Prado et al., 2014, 2011; Randak et al., 2009; Schultz et al., 2013). These proteins are synthesised in the liver of females under the regulation of 17β-oestradiol (E2), and males produce significant quantities of Vtg and Zrp upon exposure to oestrogenic endocrine disruptors (Bahamonde et al., 2014, 2013; Desforges et al., 2010; Prado et al., 2011; Tyler and Jobling, 2008). Zrp expression precedes that of Vtg, thus its expression in males is considered an early signal of the presence of oestrogenic compounds in water (Arukwe and Røe, 2008).

Besides Vtg and Zrp, insulin-like growth factors (IGFs) are targets of oestrogenic endocrine disruptors, such as E2 and 17α-ethinylestradiol (EE2), and other compounds released into the aquatic environment (Prado et al., 2014; Shved et al., 2008). IGFs are produced mainly in the liver under regulation of growth hormone and changes in expression of these proteins may affect gametogenesis and reproduction of fish species (Berishvili et al., 2006; Reinecke, 2010). Growth factors, specifically IGF-I, have various functions, such as growth regulation as well as mitogenesis, differentiation, and apoptosis inhibition in gonads (Reinecke, 2010; Shved et al., 2008).

Reproductive biomarkers such as the gonodosomatic index, fecundity, proportion of intersex fish, and sex ratio are also affected by oestrogenic EDCs (Denslow and Sepúlveda, 2007). Intersex specimens present male and female germ cells in the same gonad in gonochoristic species, and this condition can be induced by water contaminated with oestrogenic and anti-oestrogenic compounds. (Bahamonde et al., 2015; Tyler and Jobling, 2008). In South America, studies on endocrine disruption affecting the wild fish fauna are scarce and intersex gonads have been reported in few species (Chiang et al., 2015; Kinnison et al., 2000; Prado et al., 2014, 2011).

The lambari *Astyanax rivularis* (Lütken, 1875) is a small-sized, gonochoristic species of the Characidae family, inhabiting creeks and streams with strong currents and high altitudes in the upper Velhas River, São Francisco River basin (Lima et al., 2003). This species can reach up to 15 cm in total length and is omnivorous with opportunistic feeding habits, consuming allochthonous and autochthonous items (Vieira et al., 2015). The lambaris have asynchronous oocyte development, spawn in batches in a prolonged breeding season (Veloso-Júnior et al., 2009), and are suitable species to be used as a sentinel model for ecotoxicological studies (Prado et al., 2014, 2011).

The upper Velhas River is located in a transition area between the Atlantic Forest and Cerrado biomes, and both types of vegetation are considered biodiversity hotspots, due to the high number of endemic species and excessive loss of habitat (Myers et al., 2000). Despite its ecological and historical significance as one of the main routes of the gold trade in the 18th century, the Velhas River is the most polluted river in the state of Minas Gerais, due to a population of over 5 million people living in the surroundings of its basin (IBGE, 2014).

Thus, the aim of this study was to investigate the reproductive biology of *Astyanax rivularis* exposed to domestic sewage in streams of the upper Velhas River, using histological, morphometric and molecular approaches.

2. Materials and methods

2.1. Study area

The Velhas River has an average annual water flow of 631 m^3/s and drains an area of 27,867 km^2 in the central area of the state of Minas Gerais, south-eastern Brazil. The headwaters of the Velhas River, at 1520 m above sea level, are located in Andorinhas Park, municipality of Ouro Preto, a UNESCO World Heritage site for its historic buildings and culture.

For this study, three streams of the upper Velhas River were chosen for fish sampling. One stream with low anthropogenic interference (S1, reference) and two receiving municipal domestic sewage (S2 and S3) (Fig. 1). The pollution sources of each sampled stream are described in Table 1.

2.2. Fish collection

Fish collection procedures followed the ethical principles established by the Brazilian College of Animal Experimentation (COBEA), and the study was approved by the Ethics Committee on Animal Use (CEUA, protocol N° 189) of the Federal University of Minas Gerais, Brazil. A total of 1129 females and 265 males of *A. rivularis* were caught during quarterly samplings encompassing a reproductive cycle. At each sampling site, the fish were caught using 100 m (10 gill nets of 10 m) gillnets with a 0.8–1.5 cm stretched mesh size deployed for about 12 h in pools of the streams. Alive fish were euthanized with immersion in eugenol 85 $mg \cdot L^{-1}$. Total length (TL; 0.01 cm), body weight (BW; 0.01 g), gonad weight (GW; 0.001 g), and liver weight (LW; 0.001 g) were measured and the following biological indices were calculated for each fish: gonadosomatic index ($GSI = 100\ GW/BW$), liver somatic index ($LSI = 100\ LW/BW$), and Fulton condition factor ($K = 100\ BW/TL^3$).

During fish samplings, the temperature, pH, dissolved oxygen concentration, conductivity, water flow, and turbidity were measured at each collection site using a multi-parameter Horiba U51 probe, a General Oceanics flowmeter, and a Quimis turbidimeter.

2.3. Main oestrogens in the water

For analysis of the main oestrogenic endocrine disruptors, standards of oestrone (E1), oestradiol (E2), oestriol (E3), bisphenol-A (BPA), and nonylphenol (NP), were purchased from Sigma-Aldrich (St. Louis, MO, USA), all with ≥97% purity. Calibration curve, standard mass and target ions (*m/z*) of each compound are shown in Table S1 (Supplementary material). During samples collection, 500 ml surface water were obtained in amber glass, three in June (dry season) and three in December (rainy season) from each sampling site and immediately cooled to 4 °C for posterior analyses using high performance liquid chromatography (HPLC). The compounds contained in the samples were extracted by solid phase (SPE C18), dried, and eluted in 1 ml of methanol in a microcentrifuge tube. Aliquots were again dried by evaporation and resuspended in 100 μl of methanol in each tube, and were subsequently shaken in a vortex. Then, 20 μl of the samples were injected into a Shimadzu LCMS-IT-TOF (225-07100-34) liquid chromatograph equipped with a DGU-20A3 degasser, two LC-20AD pumps, a SIL-20A autosampler, a SPD-10A UV–Vis detector, and a CBM-20A communication

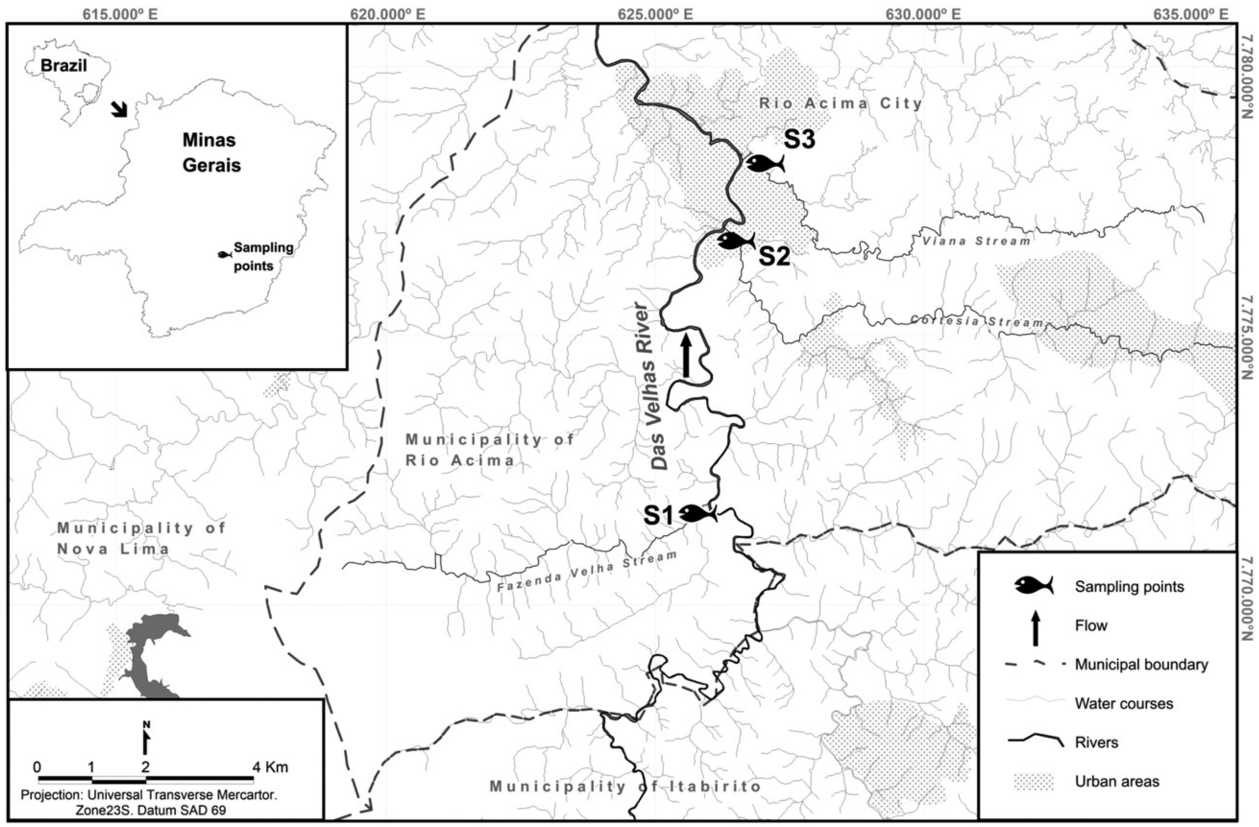

Fig. 1. Map of the Upper Velhas River, south-eastern Brazil and location of sampling sites. Reference site (S1) and impacted sites (S2 and S3).

module. A Luna pentafluorophenyl (PFP) column (100 mm, 2 mm, 3 mm - Phenomenex) was used for separation of analytes using a 0.15 ml/min flow rate under UV–Vis detector coupled with IT-TOF. After separation of the analytes in the column, they passed the first UV–Vis detector, before being sent to the mass spectrometer to confirm their identification. The mobile phase used was water (phase A) and acetonitrile (phase B). Initially, there was an isocratic elution with 70% A and 30% B for 5 min. The reverse phase chromatography was carried out by gradient, according to the following programme: from 30% B to 100% B in 30 min, hold at 100% B for 6 min, reduction to 30% B in 39 min. Mass spectrometry was carried out in MS/MS mode and the ions were monitored at +4.5 kV (positive) and −3.5 kV (negative). The monitoring range was 50–550 m/z. The accumulation time in the ion trap was 50 ms, and fragmentation was performed taking intensity as reference. The voltage at the detector and nebulizer gas flow were 1.76 kV and 1.5 l/min, respectively. The pressure of the dry gas, dry temperature, and the collision gas pressure were 130 kPa, 200 °C, and 45 kPa, respectively.

2.4. Histology and morphometry

In order to assess the gonadal maturity stages, gametogenesis, intersex and gender confirmation, histological analyses were performed for all fish caught. Samples of the middle region of the left gonad of each fish were fixed in Bouin's fluid for 8 to 12 h and then kept in 70% ethanol for histological processing. Gonad samples were gradually dehydrated in ethanol, embedded in paraffin, sectioned at 5 μm thickness, and stained with haematoxylin-eosin (HE). The gonadal maturity stages were established based on the gonadal macroscopic and microscopic features: (1) resting, (2) maturation, (3) mature, and (4) spawned (Carvalho et al., 2009). The sex ratio (females: males) was assessed at each collection site, following sex confirmation by histology.

For morphometric analyses and fecundity 15 females in stage 3 from each site were chosen randomly at the gonadal maturation peak (June), a critical developmental window for EDCs action in adults. Morphometric analysis of gonadal maturation was carried out on histological sections of gonads in stage 3 (n = 15 fish/site), collected during the maturation peak (June). For females, ovarian follicles in each developmental stage (perinucleolar, cortical alveoli, and vitellogenic) were counted and the proportion (%) of each follicle type was determined for each site. The following histopathological alterations were assessed: over-ripening, yolk deficient oocyte, and atretic follicles. The proportion of each alteration was determined in relation to the total number of vitellogenic follicles of the slide. For each site, the diameter of mature vitellogenic follicles (n = 250/site) was measured using 15 histological sections.

Table 1
Characteristics and pollution sources of three sampling sites from the upper Velhas River, Brazil.

Sampling sites	Characteristics and impacts
S1 (20°08′50″S; 43°47′43″O)	Reference site with little anthropogenic interference, localized 10 km from urban areas. Width stream ranging from 2.25 a 4.68 m and depth from 22.00 a 93.00 cm.
S2 (20°06′00″S; 43°47′33″O)	Site exposed to sewage discharges from households and breeding of domestic animals including pig, without any treatment. Width ranging from 4.20 to 7.20 m and 15.00 to 81.00 cm depth.
S3 (20°05′17″S; 43°47′12″O)	Site located within urban areas, receiving sewage untreated households and homes. This stream has a width between 3.21 and 7.70 m and depth 37.00 to 88.00 cm.

For males, proportion (%) of the germ cells and diameter of the seminiferous tubules (ST) were determined using 10 fish from each site. In each histological section, diameter of 10 ST was measured, totalling 100 ST/site. From each histological slide, five images at 400× magnification were obtained. The images were overlaid a 26 × 19 point grid (494 intersections points) using the Image J software, and a total of 24,700 points were analysed per site. The proportion of spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa was obtained for each image. Other testicular components (myoid cells, connective tissue, Leydig cells, Sertoli cells, and immune cells) were grouped as somatic cells. Tubular intersections that did not reach any cell were included in the white spaces category.

2.5. Fecundity

Ovaries of 15 mature females caught at the maturation peak (June) were chosen randomly to estimate fecundity for each site. Samples of the middle region of the right ovary were weighed and kept in Gilson's solution (100 ml of 60% ethanol, 880 ml distilled water, 15 ml of 80% nitric acid, 18 ml glacial acetic acid, and 20 g of mercuric chloride) until complete dissociation of the oocytes. Batch fecundity (BF = OVA × GW) and relative batch fecundity (RBF = BF/TL) were estimated considering the number of mature oocytes per gram of ovary (OVA), the ovary weight (GW) and the total length (TL).

2.6. Biomarkers Vtg, Zrp and IGF-I

During the samplings, liver samples (n = 10/sex/site) of mature fish caught alive in the maturation peak (June) were stored in liquid nitrogen and then kept in a freezer at −80 °C. The samples were subjected to ELISA assay for Zrp, Vtg, and IGF-I. The frozen samples were homogenised in extraction buffer (50 mM Tris-HCl pH 8.0 with 0.002% aprotinin and 1 mM phenylmethylsulfonyl) at a 1:2 ratio of tissue weight: buffer volume using a Potter S (Braun, Melsungen, Germany) homogeniser. Subsequently, the extracts were sonicated using a GEX 600 EC ultrasonic processor and then centrifuged at 15,000*g* for 60 min at 4 °C. After centrifugation, the supernatants were stored in aliquots at −80 °C until analysis. Total protein contained in the liver homogenates was determined by the Bradford method using bovine serum albumin (BSA) as standard. Duplicate samples of 100 μg/ml (Vtg and Zrp) and 500 μg/ml (IGF-I) were incubated overnight in microplates with 96 wells (Nunc, Denmark), blocked with 2% BSA, and incubated again at 37 °C for 2 h with rabbit polyclonal primary antibody at 1:500 dilution for anti-salmon Vtg and Zrp (Biosense Laboratories AS, Norway) and anti-human IGF-I (Santa Cruz Biotechnology, Inc., USA). After washing with PBS-Tween 0.05%, the plates were incubated with anti-IgG secondary antibody (1:1000, Sigma, St. Louis, MO) conjugated with peroxidase for 2 h at 37 °C. After washing, the reaction was revealed with 200 μl of o-phenylenediamine dihydrochloride (OPD) (Sigma, St. Louis, MO, USA) solution in 0.05 M phosphate-citrate buffer containing 0.0025% hydrogen peroxide. The reaction was stopped by adding 50 μl of 5% H_2SO_4 to each well and the absorbance was measured at 492 nm using a BioTek Instruments Inc. spectrophotometer. For validation of the ELISA assays, dilution curves of liver homogenates were performed for each protein analysed (Fig. S1, Supplementary material).

The specificity of the antibodies used in the ELISA assay was determined by Western blotting, using 100 μg of sample (n = 5 females from S1) added to the 7.5% (Vtg), 10% (Zrp), and 15% (IGF-I) electrophoresis gel (SDS-PAGE) (Fig. S1, Supplementary material). The proteins were transferred to nitrocellulose membranes and then subjected to blocking of nonspecific reactivity with 2% casein. After blocking, the membranes were incubated with the primary antibodies (anti- Zrp, anti-Vtg and anti-IGF-I) at a dilution of 1:500, washed with PBS with Tween 20 buffer, and incubated for 10 min with secondary antibody conjugated with peroxidase (anti- IgG Sigma, 1: 1000). The reaction was revealed by the addition of 3,3′-diaminobenzidine (DAB) in PBS containing 4-chloro-1-naphthol, methanol, and hydrogen peroxide.

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism software version 5.0 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Values are expressed as means ± SEM, and the results were considered significant at a 95% confidence interval. Annual means of the physico-chemical parameters of the water were compared among sites by One-way variance analysis (ANOVA) followed by Tukey's post-test. As the biological data and oestrogenic compounds in water did not show a normal distribution, they were analysed using the nonparametric Kruskal-Wallis test, followed by Dunn's post-test. In order to detect deviations in the expected sex ratio of 2 females to 1 male (Carvalho et al., 2009) from each sampling site, a chi-square test (χ^2) was used.

3. Results

3.1. Water quality

In the upper Velhas River, the water temperature ranged from 14 to 21 °C, and the dissolved oxygen from 8 to 15 mg/l, with no significant differences between sampling sites ($p = 0.07$ and $p = 0.66$, respectively) (Table 2). Conductivity ($p < 0.0001$), water flow ($p = 0.0007$), and turbidity ($p < 0.0001$) were higher in the reference site S1, while the pH was significantly higher in S2 and S3 ($p < 0.0001$) (Table 2).

Analyses of the oestrogenic compounds in the water at the reference site, S1, showed concentrations <70 ng/l for all substances analysed (Table 3). Mean values of oestrone (E1) were significantly higher in S2 (~250 ng/l) compared to S1 and S3 (~10 and 33 ng/l, respectively). Oestriol (E3, ~200 ng/l), bisphenol A (~200 ng/l), and nonylphenol (~650 ng/l) were higher in S3 compared to S1 and S2. High levels of oestradiol (E2, ~200 ng/l) were observed in the two sites exposed to sewage, S2 and S3.

3.2. Biological indices, fecundity, and sex ratio

Considering the biometric data and the biological indices (Table 4), the males and females were larger and heavier in S1 ($p < 0.0001$). The highest GSI values were observed in S3 females ($p < 0.0001$), but there was no significant difference for males ($p = 0.192$). LSI was significantly higher in S2 and S3 for females ($p < 0.0001$) (Table 4), these variations occurred mainly in December ($p < 0.0001$) and March ($p < 0.0002$) (Fig. 2c). In males, LSI was higher in December ($p = 0.01$) and June ($p = 0.0013$) in fish from S2 (Fig. 2d). Overall, Fulton condition factor (K) was higher in S2 and S3 for both females and males ($p < 0.0001$) (Table 4), however few significant variations were found among sampling periods (Fig. 2e and f). Lower values of batch fecundity were observed in S2 (1920 ± 639 eggs) compared to S3 (2886 ± 1237 oocytes) (Table 4). The sex ratio in S1 (2.2 F: 1 M; $p = 0.38$) was similar to the expected (2 females: 1 male), but an

Table 2
Water physic-chemical parameters in three sampling sites from the upper Velhas River, Brazil, during 2014 and 2015.

	S1	S2	S3
Temperature (°C)	18.05 ± 2.28^{a}	17.52 ± 1.76^{a}	17.56 ± 1.79^{a}
Dissolved oxygen (mg/L)	11.59 ± 2.09^{a}	11.35 ± 2.07^{a}	11.65 ± 1.67^{a}
pH	6.55 ± 0.39^{a}	6.77 ± 0.31^{b}	6.70 ± 0.37^{b}
Conductivity (μS/cm)	56.65 ± 0.29^{c}	33.18 ± 0.95^{b}	17.15 ± 0.34^{a}
Water flow (cm/s)	56.61 ± 18.02^{b}	36.41 ± 18.73^{a}	37.78 ± 17.49^{ab}
Turbidity (NTU)	13.79 ± 6.62^{b}	6.69 ± 3.68^{a}	5.15 ± 4.20^{a}

Values represent mean ± SEM of data obtained during four samplings. Different letters indicate significant differences among sites, ANOVA One-Way, $p < 0.05$.

Table 3
Water environmental oestrogens (ng/L) at three sampling sites from the upper Velhas River, Brazil.

	S1	S2	S3
Oestrone (E1)	10.17 ± 6.48[a]	256.66 ± 87.24[b]	33.33 ± 15.02[a]
Oestradiol (E2)	46.17 ± 17.47[a]	216.67 ± 44.91[b]	203.33 ± 63.05[b]
Oestriol (E3)	50.00 ± 13.44[a]	147.83 ± 90.03[ab]	211.50 ± 72.35[b]
Bisphenol A (BPA)	34.61 ± 15.54[a]	97.14 ± 41.87[ab]	198.66 ± 61.83[b]
Nonylphenol (NP)	67.20 ± 15.18[a]	263.11 ± 200.83[a]	645.01 ± 408.73[b]

Values represent mean ± SEM (n = 6 samples). Different letters indicate significant differences among sites, Kruskal-Wallis, p < 0.05.

accentuated deviation was observed in S2 (6.5 F: 1 M; p < 0.0001) and S3 (3.2 F: 1 M; p = 0.0001) (Table 4).

3.3. Gonadal maturation

For both males and females of *A. rivularis*, lower GSI were found in December and March, and the higher values occurred in September and June (Fig. 2a and b). Comparing the sites, females GSI was significantly higher in September (p = 0.03) and June (p < 0.0001) in S3 in relation to S2. In December, the GSI was statistically higher in S1 for females and males, when compared to S2 and S3 (p = 0.03), and there were no significant differences in March (Fig. 2a and b).

Annual variations of LSI were evident in females, values being significantly higher in S1 and S2 during September and June (p < 0.05) (Fig. 2c), periods of higher GSI and high frequency of mature females (stage 3). Otherwise, LSI of males exhibited no significant variations (p > 0.05) (Fig. 2d) while K showed few differences throughout the periods at the different sampling sites (Fig. 2e and f).

The frequency of gonadal maturation stages showed females and males at resting (stage 1) mainly in December and March (Fig. 3). Gametogenesis and gonadal maturation (stages 2 and 3) were more abundant in June and September, while spawning (stage 4) occurred in September, December, and March. Comparing the sampling sites, more mature females (stage 3) were recorded in June in S3 (74%) compared to S1 (54%) and S2 (49%). In the months of December and March, a higher number of females at resting was observed in S2 (71% and 62%, respectively) and S3 (81% and 36%, respectively) compared to specimens from S1 (48% and 50%, respectively). Regarding the males, in June, mature specimens (stage 3) were abundant (88% in S1, 91% in S2, and 74% in S3). In December, 41% of males from S1 were at resting (stage 1) while for S2, 67% of all specimens were at resting. No male from S3 was caught in March.

Table 4
Biological and reproductive parameters of *Astyanax rivularis* at three sampling sites, from the upper Velhas River, Brazil.

		S1	S2	S3
Females	N	167	688	274
	TL (cm)	11.05 ± 0.90[c]	9.60 ± 0.91[a]	9.94 ± 1.00[b]
	BW (g)	18.56 ± 4.77[c]	12.71 ± 3.72[a]	14.20 ± 4.03[b]
	GSI	4.24 ± 0.35[a]	4.44 ± 0.19[a]	10.52 ± 0.37[b]
	LSI	1.16 ± 0.64[a]	1.47 ± 0.68[b]	1.44 ± 0.68[b]
	K	1.36 ± 0.19[a]	1.41 ± 0.18[b]	1.42 ± 0.22[b]
	BF (10^2)	25.93 ± 10.82[ab]	19.20 ± 6.39[a]	28.86 ± 12.37[b]
	BF/TL(10^2)	2.32 ± 0.91[ab]	1.79 ± 0.57[a]	2.76 ± 1.07[b]
Males	N	74	105	86
	TL (cm)	9.32 ± 0.08[a]	8.53 ± 0.05[b]	8.36 ± 0.06[b]
	BW (g)	11.04 ± 0.38[a]	8.88 ± 0.17[b]	8.73 ± 0.19[b]
	GSI	7.28 ± 0.51[a]	7.16 ± 0.55[a]	8.46 ± 0.46[a]
	LSI	1.03 ± 0.04[a]	1.24 ± 0.05[b]	0.94 ± 0.04[a]
	K	1.34 ± 0.02[a]	1.43 ± 0.02[b]	1.50 ± 0.02[c]
Sex ratio (F:M)		2.2:1	6.5:1*	3.2:1*

Values represent mean ± SEM. Different letters indicate significant differences among sites, Kruskal-Wallis, p < 0.05. (TL) total length; (BW) Body weight; (GSI) gonadosomatic index; (LSI) Liver somatic index; (K) Fulton condition factor; (BF) Batch Fecundity; (BF/TL) Relative batch fecundity, * indicates significant differences, chi-square test, p < 0.05.

3.4. Gametogenesis

A larger diameter for the vitellogenic follicles (p = 0.0002), a higher proportion of perinucleolar follicles (p = 0.0003), and a lower ratio of vitellogenic follicles (p = 0.0003) were found in S2 (Table 5). No statistical difference was observed between the sampling sites in the proportion (%) of follicles with cortical alveoli (p = 0.15). Most fish from S1, the reference site, showed healthy ovaries and testis (Fig. 4A and B). Over-ripening (oocytes ageing with increased follicular diameter, basophil granules accumulated in the peripheral ooplasm, formation of perivitelline space, and thicker zona radiata) were more frequent in S3 (p = 0.005) (Fig. 4C). Yolk deficient oocytes (changes in morphology and integrity of the yolk globules especially in the peripheral ooplasm) were more abundant in S2 and S3 females compared with S1 (p = 0.024) (Fig. 4D and E). The frequency of atretic follicles (follicles with yolk liquefaction, fragmentation of the zona radiata, and follicular cell hypertrophy) was low in the three sites, without significant difference (p = 0.665) (Table 5). Intersex gonads had a multifocal distribution of perinucleolar follicles arranged randomly in the testicular parenchima (Fig. 4F). Intersex fish were caught in December and June in S2 (6 and 8%, respectively) and June in S3 (15%) (Fig. 3d and f), but they were identified only in the histological analyses of their gonads. No intersex was found in site S1 (Fig. 3b).

The diameter of the seminiferous tubules was significantly smaller in males from S2 compared to S1 and S3 (p < 0.0001). A higher proportion (%) of spermatogonia was found in S2 (p = 0.0001). In S3, there was a higher proportion of primary spermatocytes (p = 0.0054) and spermatids (p < 0.0001) compared to S1. Spermatozoa were significantly more abundant in S1 when compared to S2 and S3 (p = 0.0001) (Table 6).

3.5. Hepatic levels of Zrp, Vtg, and IGF-I

In females, the highest values of ELISA absorbance for Zrp were detected in samples from S2 compared to S1 and S3 (p < 0.001) (Fig. 5a). The Vtg levels were statistically lower in S3, compared to S1 (Fig. 5c). No statistical difference was detected for IGF-I in females from the different collection sites (Fig. 5E).

In males, the highest hepatic Zrp values were observed in S2 and S3, but no significant difference was detected among sites (p = 0.87) (Fig. 5b). Hepatic Vtg levels in males from S2 and S3 were significantly greater than S1 (p = 0.0024) (Fig. 5D). IGF-I levels were statistically lower in males from S2, when compared to the values observed in S1 (p = 0.03) (Fig. 5F).

Corroborating the endocrine disruption in *A. rivularis*, hepatic levels of Zrp and Vtg in females from S1 were similar to males from S2 and S3. Females from site S1 and males from sites S2 and S3 presented significantly higher Vtg levels than males from site S1 (p = 0.0017). No statistical difference was observed between Zrp levels of the males of the three sites and the females from site S1 (p = 0.12).

4. Discussion

Field studies that assessed the effects of oestrogenic contaminants on reproductive biology in a sentinel fish species using molecular, cellular, and ecological approaches are scarce (Chiang et al., 2015; Prado et al., 2014, 2011). Accordingly, this study reports data on multiple parameters of the reproductive biology (Vtg, Zrp, IGF-I, GSI, intersex, gametogenesis, fecundity, and seasonal variations of gonadal maturity, spawning season, sex ratio, and others) in the lambari *A. rivularis* from three streams of the upper Velhas River. These data support that exposure of fish to oestrogenic mixtures affects reproductive biomarkers, ranging from molecular to population level (Denslow and Sepúlveda, 2007), that which may disturb the sustainability of wild fish.

High concentrations of natural (E1, E2, and E3) and synthetic (NP and BPA) oestrogens were detected in two impacted streams

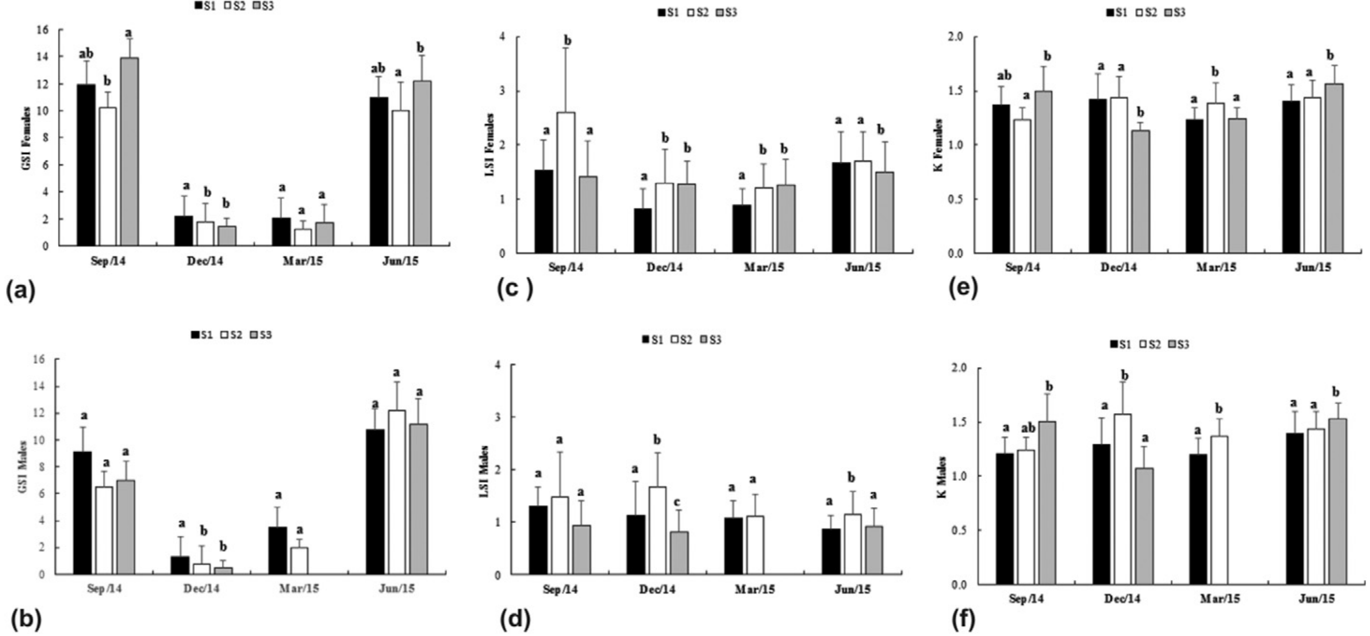

Fig. 2. Seasonal variations of gonadosomatic index (GSI) for females (a) and males (b), liversomatic index (LSI) for females (c) and males (d), Fulton condition factor (K) for females (e) and males (f) of *Astyanax rivularis* caught in the sampling sites: reference site (S1) and impacted sites (S2 and S3). Different letters indicate significant differences between sites, Kruskal-Wallis, $p < 0.05$.

of the upper Velhas River: natural (20–750 ng/l) and synthetic (59–1439 ng/l). The high oestrone (E1) values found in S2 may be associated with pig farming in adjacent areas to this site, since this is the predominant oestrogen during late pregnancy in pigs (Grzesiak et al., 2014). Concentrations of nonylphenol (NP), ranging from 25.9 to 1435.3 ng/l, and bisphenol A (BPA), varying between 8.6 and 168.3 ng/l, were detected in the main course of the upper Velhas River (Moreira et al., 2011). These synthetic oestrogens are released from plastic products, which are discarded into the rivers. In the present study, more elevated concentrations of 17β-oestradiol (E2) were found in the contaminated sites, S2 and S3 (100–480 ng/l), than in the main course of the Velhas River (0–62 ng/l) (Moreira et al., 2011). Indeed, studies have shown that the concentrations of natural oestrogens are higher in smaller water courses, and gradually decrease as the water volume of the tributaries increases until reaching the main river (Nie et al., 2014). Furthermore, each oestrogenic compound has distinct degrees of persistence in the aquatic environment, and some can be degraded in a few days, such as 17β-oestradiol (E2), while others persist for up to 150 days, such as bisphenol A (Lintelmann et al., 2003). Water bodies far from urban centres and agricultural activities have low concentrations of oestrogenic endocrine disruptors, which was observed in the reference site S1 of this study, presenting average values <70 ng/l for all substances analysed. In this sense, mean concentration of E2 (46 ng/l) as well as other oestrogens at site S1 were lower than in the main course of the Velhas River, corroborating S1 as a reference site. The presence of oestrogens in aquatic environments is an environmental problem commonly observed in the rivers and lakes of several countries (Atkinson et al., 2012; Bahamonde et al., 2015; Kidd et al., 2007; Prado et al., 2014; Schultz et al., 2013; Sim et al., 2011). Likewise, this complex situation is also affecting fish communities in Brazilian rivers, and it needs to be managed urgently for the conservation of the environment and biodiversity.

Presence of trace metals were detected in sites of natural preservation in the upper Velhas River basin and >70% of the sediment from streams showed exclusively natural concentration ranges for the metals studied (Vicq et al., 2015). In addition, the waters of the Velhas River are slightly acidic (Pompeu et al., 2005) as well as observed for all sampling sites. Therefore, other factors are also influencing the water quality parameters especially conductivity and turbidity in the upper Velhas River. In fact, this area is located in a region with great concentration of iron, denominated Iron Quadrangle in Minas Gerais, Brazil. Furthermore, the water flow in S1 may be associated with the geology of this site. In fact, *A. rivularis* is adapted to places with rapids and great water oxygenation (Veloso-Júnior et al., 2009).

Chronic exposure to oestrogenic compounds can cause impairment in the somatic growth, leading to biometric changes in fish populations (Reinecke, 2010). In present study females and males from S2 and S3 had lower length and weight, demonstrating growth deficiency, as was also observed in species submitted to oestrogenic contamination (Arsenault et al., 2004; Silva et al., 2012). We would expect lower IGF-I on these sites, but surprisingly hepatic expression of IGF-I was lower in males from S2 only, indicating that other biomarkers should be considered to assess the effects of oestrogenic EDCs in environment on somatic growth in *A. rivularis*. In tilapia, exposure during early development to EE2 led to growth impairment and feminization and altered IGF-I expression not only in liver but also in gonads (Shved et al., 2008). Since teleosts exhibit a range of growth traits and regulatory mechanisms further studies are necessary to clarify the effects of EDCs on somatic growth in teleost fish.

In addition to fish size, endocrine disrupters also affect biological indices (Denslow and Sepúlveda, 2007). However, the absence of significant differences in the GSI of males and higher values in this index of females from site S3 reveals that the use of this index alone does not reflect the real effects of the toxic on the fish gonad (Silva et al., 2012). Field studies with *Astyanax fasciatus* have shown increased LSI in individuals collected near agricultural, industrial, and urban areas (Prado et al., 2014, 2011), similar to that observed in fish from S2 and S3 in this study. The higher LSI values can be related to hypertrophy of the organ for toxin removal as well as accumulation of lipids and protein synthesis induced by exposure to oestrogenic compounds (Fishelson, 2006). Moreover, cyclical variations of LSI may also reflect the protein synthesis in liver, mainly Vtg and Zrp during gonadal maturation (Denslow and Sepúlveda, 2007). Indeed, LSI variations in *A. rivularis* were more evident in females than males, especially in S1 and S2, values being higher in September and June, periods of high GSI and frequency of mature females.

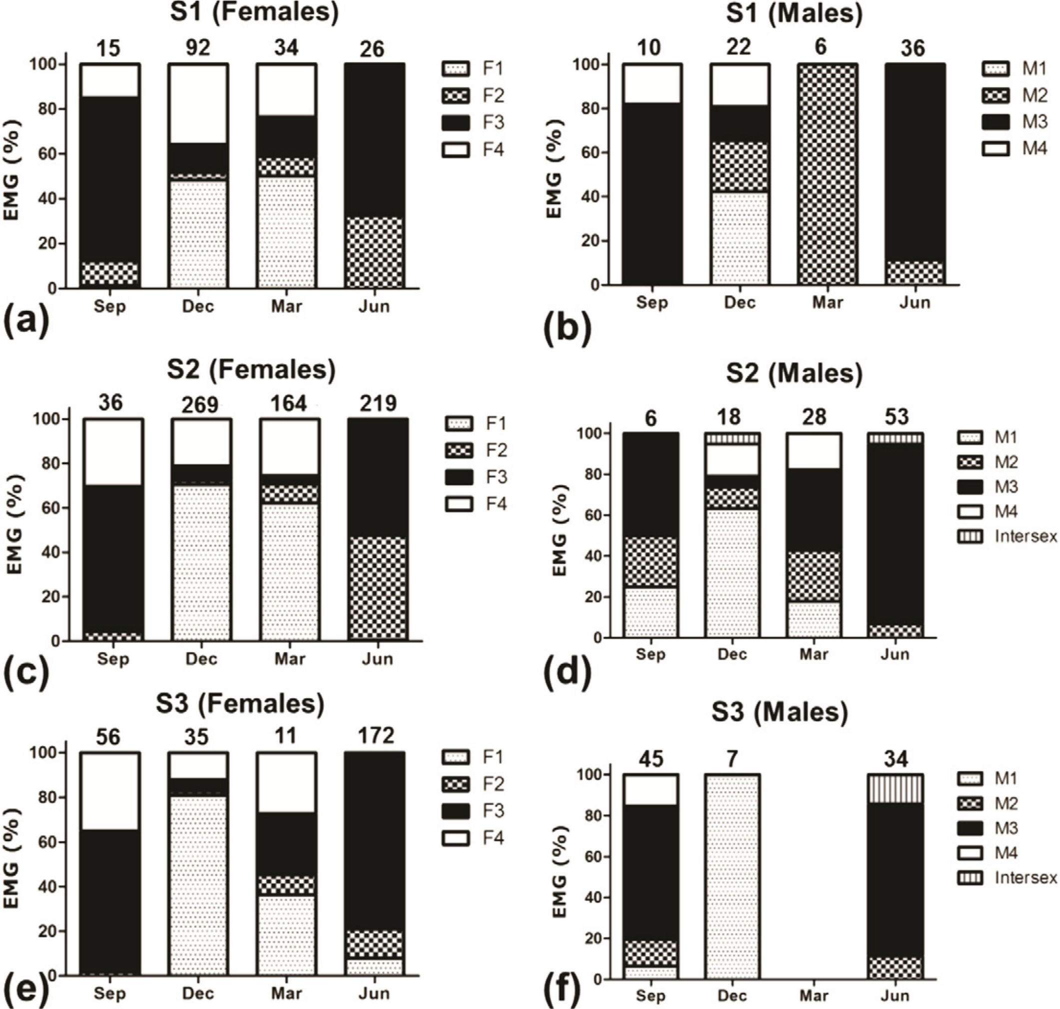

Fig. 3. Seasonal distribution of the frequencies (%) of the gonad maturation stages for females and males of *Astyanax rivularis* caught at three sites from the Upper Velhas River southeastern Brazil, from September 2014 to June 2015. Gonad maturity stages for females (F) and males (M): (1) resting, (2) maturation, (3) mature, and (4) spawned. The numbers above the columns indicate the fish abundance collected in each sampling period.

Fulton condition factor (K) reflects the physiological condition of the fish in its habitat through the accumulation of energetic reserves (Froese, 2006). In general, females exhibit higher values of K during gonadal maturation and a reduction after spawning (Carvalho et al., 2009). In the present study, variations of K were few evident through the sampling periods since *A. rivularis* spawns in batches in a prolonged breeding season (September to March). Study carried out with *Gobio gobio* population located in an impacted environment exhibited higher K values (Knapen et al., 2009) such as found in S2 and S3 in this study. It is important to note that intersex individuals, who live in sites contaminated by oestrogenic compounds, have also higher K values (Bahamonde et al., 2015). These observations indicate a change in the weight-length ratio in the contaminated sites by EDCs. Indeed, condition factor is an important biomarker in studies evaluating the effects of aquatic contamination and the implications for adaptation of populations inhabiting polluted environments (Venâncio and Domingos, 2014).

Table 5
Vitellogenic follicles diameter (μm) and proportion (%) of ovarian follicles in *Astyanax rivularis* from three sampling sites, upper Velhas River, Brazil.

	S1	S2	S3
Vitellogenic follicles diameter	865.14 ± 85.04[a]	901.59 ± 87.88[b]	831.05 ± 131.39[a]
Perinucleolar follicles	49.1 ± 2.9[a]	69.4 ± 2.5[b]	57.1 ± 3.5[a]
Cortical alveoli	3.4 ± 0.5[a]	2.5 ± 0.4[a]	2.2 ± 0.6[a]
Vitellogenic follicles	47.5 ± 3.5[b]	28.1 ± 3.5[a]	40.7 ± 3.5[b]
Healthy vitellogenic follicles	96.84 ± 1.72[b]	92.4 ± 2.72[b]	82.12 ± 4.63[a]
Follicular atresia	1.13 ± 0.28[a]	1.15 ± 0.31[a]	1.94 ± 0.51[a]
Yolk deficient follicles	0.39 ± 0.20[a]	5.32 ± 2.17[b]	6.34 ± 3.30[b]
Over-ripened follicles	1.64 ± 0.89[a]	1.13 ± 0.46[a]	9.60 ± 5.25[b]

Values represent mean ± SEM of data obtained from 15 females caught in June (maturation peak). S1, reference site, S2 and S3, sites contaminated with estrogenic compounds. Different letters indicate significant differences among sites, Kruskal-Wallis, $p < 0.05$.

A deviation in sex ratio can be a consequence of exposure to EDCs during the early stages of development and may be associated with the contamination of the aquatic environment by oestrogenic (deviation for females) or anti-oestrogenic (deviation for males) compounds (Holbech et al., 2006; Luzio et al., 2016, 2015). The expected sex ratio (2F: 1M) for species of the genus *Astyanax* (Carvalho et al., 2009) was observed in the reference site S1, while accentuated deviations in the proportion of females to males occurred in S2 (6.5F: 1M) and S3 (3.2F: 1M). Moreover, exposure of adult fish to oestrogenic compounds during gonadal maturation can lead to the intersex condition (Kidd et al., 2007), such as found in fish from S2 and S3. Intersex males captured in this study had perinucleolar follicles (primary growth), as also observed in *Rutilus rutilus* in the rivers of Denmark (Bjerregaard et al., 2006). However, studies with *A. fasciatus* in a reservoir in Brazil found intersex males with vitellogenic follicles occupying a large part of the testis (Prado et al., 2011). The presence of vitellogenic follicles in the gonads of males demonstrates a higher degree of intersex severity

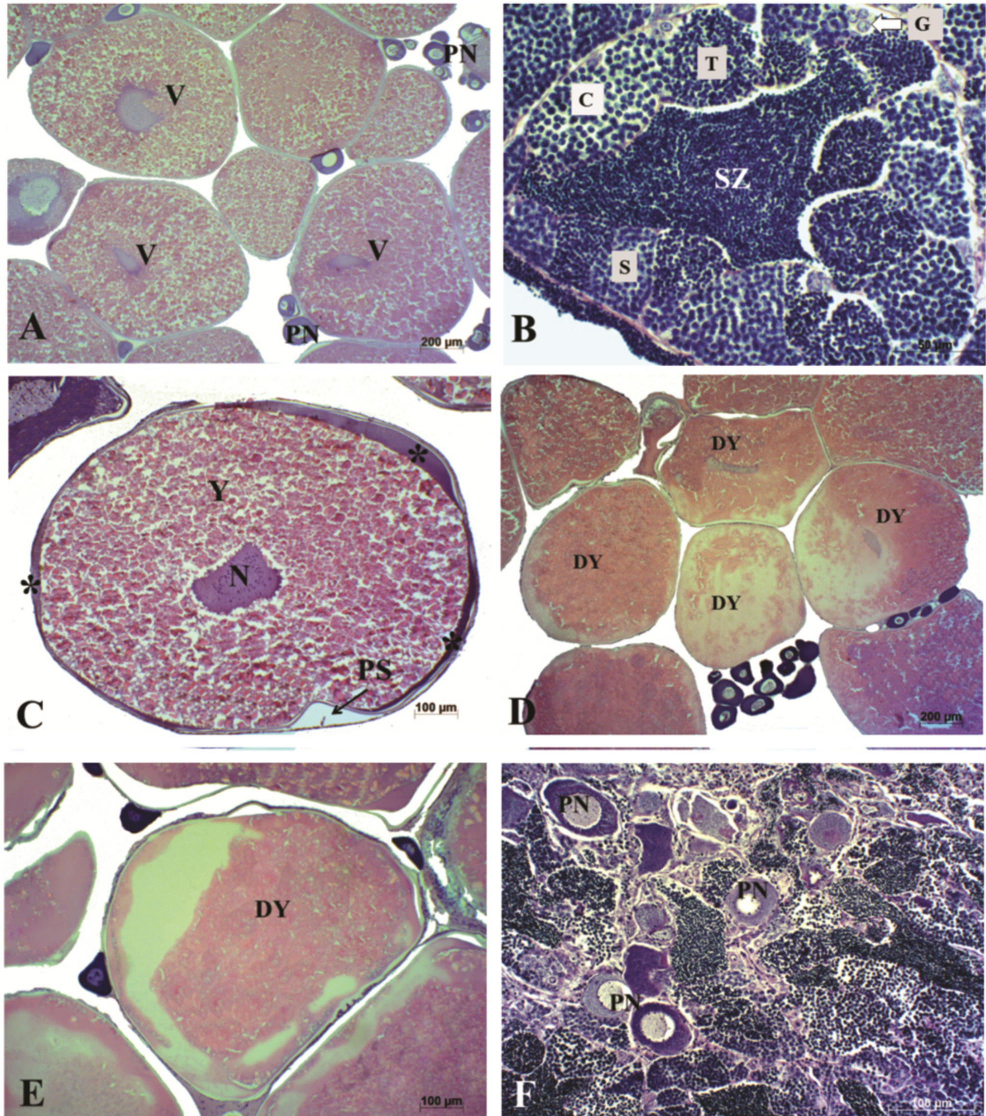

Fig. 4. Histological sections showing gonad maturation of *A. rivularis*. (A) Perinucleolar (PN) and vitellogenic (V) follicles from female caught at site S1 and (B) seminiferous tubules with spermatogonia (G), primary spermatocytes (Cy), secondary spermatocytes (S), spermatids (T), and spermatozoa (Z) from male collected at site S1; (C) over-ripening (ageing oocyte) from female at site S3, with basophilic material accumulated in the peripheral ooplasm (*) and formation of periviteline space (PS), central nucleus (N) and yolk (Y); (D) yolk deficient oocytes from female at site S2 (DY); (E) Detail of yolk deficiency in the peripheric ooplasm; (F) intersex male exhibiting multifocal distribution and only perinucleolar follicles (PN) in testicular tissue from site S3.

(Bahamonde et al., 2015), which may affect reproductive success and, in some cases, lead to a population collapse (Harris et al., 2011; Kidd et al., 2007).

Table 6
Seminiferous tubules (ST) diameter (μm) and proportion (%) of spermatogenic cells in *Astyanax rivularis* from three sampling sites, upper Velhas River, Brazil.

	S1	S2	S3
ST diameter	561.09 ± 154.72^b	436.46 ± 119.06^a	517.66 ± 141.67^b
Spermatogonia	9.08 ± 1.34^a	14.68 ± 1.40^b	7.89 ± 0.72^a
Early spermatocytes	11.92 ± 1.92^a	14.56 ± 1.27^{ab}	18.09 ± 1.66^b
Late spermatocytes	6.56 ± 1.10^a	7.34 ± 0.83^a	7.05 ± 0.64^a
Spermatids	5.97 ± 0.74^a	7.71 ± 0.61^a	11.92 ± 1.06^b
Spermatozoa	50.84 ± 3.73^b	34.92 ± 2.77^a	30.03 ± 3.01^a
Somatic cells	7.16 ± 0.91^a	6.04 ± 0.80^a	5.47 ± 0.46^a
White spaces	8.48 ± 0.92^a	14.75 ± 1.22^b	19.55 ± 1.24^c

Values represent mean ± SEM of data obtained from 10 males caught in June (maturation peak). S1, reference site, S2 and S3, sites contaminated with estrogenic compounds. Different letters indicate significant differences among sites, Kruskal-Wallis, $p < 0.05$.

Besides the intersex males, the presence of oestrogenic endocrine disruptors in the impacted sites of the present study caused changes in the gametogenesis of *A. rivularis*. In S2, females had a lower proportion of vitellogenic follicles and larger proportion of perinucleolar follicles, similar to the results obtained with *Danio rerio* experimentally exposed to EE2 (Luzio et al., 2015; Silva et al., 2012). In rainbow darter *Etheostoma caeruleum*, a lower proportion of perinucleolar follicles was found in areas of the Grand River, Ontario, Canada, impacted by oestrogenic EDCs (Bahamonde et al., 2014). Also, in males from impacted sites in the present study had a higher amount of spermatogonia, spermatocytes, and spermatids but a lower spermatozoa ratio at gonadal maturation peak, similar to the results obtained with *Danio rerio* experimentally exposed to EE2 (Silva et al., 2012). The high proportion of spermatogonia in males from S2 may be associated with the high concentration of natural oestrogens found in this site, since spermatogonial proliferation can be triggered by them (Miura et al., 1999). These data indicate that oestrogenic endocrine disruptors can affect the dynamics of gametogenesis in different ways depending on the toxin and

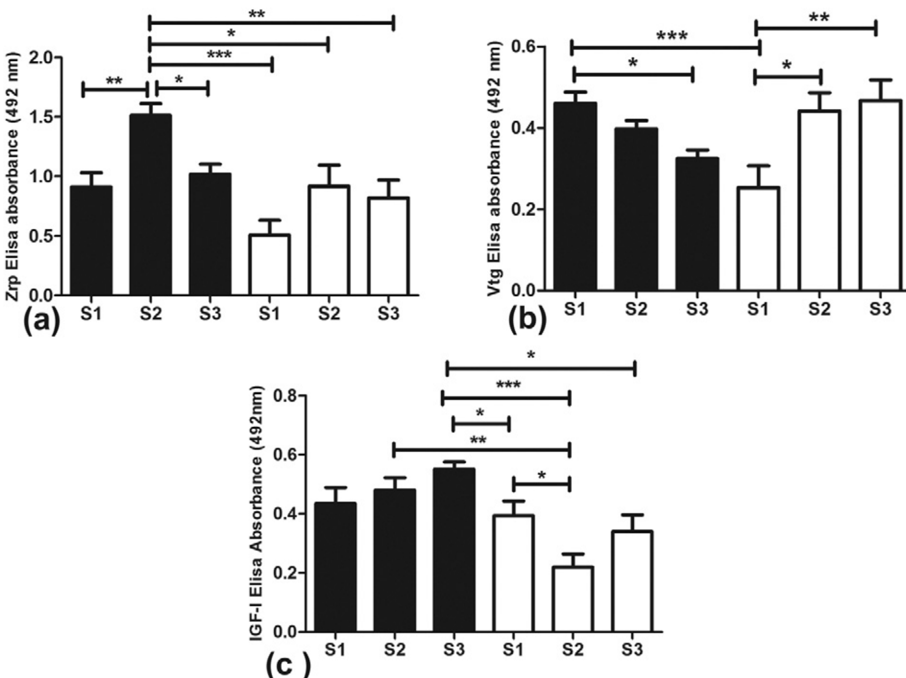

Fig. 5. Hepatic levels of zona radiata proteins (Zrp) (a), vitelogenin (Vtg) (b), and insulin-like growth hormone (IGF-I) (c) of *A. rivularis* in three sites from the upper Velhas River, south-eastern Brazil. (■) females and (□) males. Data are expressed as mean ± standard error (n = 10/sex/site) of indirect ELISA absorbance values at 492 nm. Significant differences between the sites are marked with asterisk(s), *p < 0.01; **p < 0.001; ***p < 0.0001.

level of water contamination and reproductive strategies of the species.

In females, the constant release of oestrogenic compounds into the aquatic environment promotes a delay in the steroidogenic shift of 17β-oestradiol into maturation inducing steroid (MIS), responsible for the final oocyte maturation and spawning (Noaksson et al., 2005). This delay leads to the ageing of oocytes, making them over-ripe (Flett et al., 1996). In the present study, females from S3 had elevated GSI values in June (gonad maturation peak) and about 10% of them exhibited evidence of over-ripening. Likewise, females of *A. fasciatus* showed a high proportion (33–35%) of over-ripened oocytes in the Furnas reservoir, Grande River, south-eastern Brazil (Prado et al., 2014). High rates of over-ripening lead to impairment of egg viability after fertilisation and may compromise the sustainability of fish populations in sites impacted by oestrogens.

Physiological follicular atresia can occur at any stage of the reproductive cycle by regulating the recruitment of follicles for maturation and higher rates (5 to 20%) are found only after spawning (Arantes et al., 2010; Santos et al., 2008). Yolk liquefaction, zona radiata breakdown, yolk engulfment by the follicular cells, autophagy and apoptosis are events often observed during follicular atresia in fish ovary (Morais et al., 2012). In the present study, the rate of follicular atresia was low in the three sites (<5%), but other alterations were more frequently observed in ovaries of *A. rivularis* such as yolk deficient oocytes and over-ripening (oocyte ageing) than follicular atresia. On the other hand, an increase in follicular atresia was detected in *Barbatula barbatula* in rivers from Belgium affected by sewage treatment plants (Douxfils et al., 2007), and in zebrafish *Danio rerio* submitted to EE2 (Silva et al., 2012). These differences may be related to the different regression mechanisms regulating follicular atresia in fish ovaries.

ELISA assay detected increased expression of hepatic Vtg in males from S2 and S3, sites exposed to domestic sewage, and the levels obtained were similar to females from S1, reference site. Field and laboratory studies reported an increase in Vtg levels in males of several fish species exposed to oestrogens, Vtg being a widely used biomarker to assess endocrine disruption (Bahamonde et al., 2014; Prado et al., 2014; Randak et al., 2009; Schultz et al., 2013). Moreover, some studies have shown that in sites with high estrogenic contamination the production of Vtg in males may exceed the production by females (Adeogun et al., 2016). In contrast, Vtg levels in the liver of females from the impacted sites (S2 and S3) were lower than those obtained in females from reference site (S1). Interestingly, histological analyses showed a higher frequency of yolk deficient oocytes in fish from S2 and S3. In *Cyprinus carpio*, a mixture of oestrogenic compounds in high concentrations can inhibit the secretion of gonadotropins through a negative feedback process, resulting in E2 suppression, and thereby reducing Vtg production in females (Folmar et al., 1996), an effect that may have occurred in the females from S2 and S3 of this study. To our knowledge, this study reports for the first time an association between reduced hepatic Vtg levels in females and a particular histopathological alteration in ovaries (i.e. yolk deficient oocytes) in a fish species inhabiting environments contaminated with oestrogenic endocrine disruptors.

Besides Vtg, another biomarker used in oestrogenic endocrine disruption studies is the zona radiata proteins (Zrp). In the analysed sites, the highest Zrp values were found in S2, with a significant difference only for females, where high concentrations of the natural oestrogens, E1 and E2, were found. A study of a reservoir from Nigeria reported increased Zrp in fish males and females contaminated with nonylphenol, organotin, and polychlorinated biphenyls (PCBs) (Adeogun et al., 2016). Differences in the expression of the Zrp monomers (α, β, γ) were detected by Western blotting in males from different impacted sites in a reservoir in south-eastern Brazil (Prado et al., 2011). This difference may be associated to the contamination by different compounds in the aquatic environment. A laboratory study of *Salmo salar* males subjected to only one xenoestrogen contaminant led to the expression of Zrp-β, while the combination of each contaminant associate with nonylphenol induced the expression of the three monomers of Zrp (Arukwe et al., 2000). In the present study, the three Zrp monomers were detected in females from S1 by Western blotting.

Despite the alterations detected in the reproductive parameters and EDC biomarkers, the frequencies distribution of the reproductive cycle stages associated to seasonal variations of GSI showed that *A. rivularis* reproduces in the three streams of the upper Velhas River, with batch spawning occurring from September to March. In fact, the lambaris

are widely distributed in South American river basins, and are frequently found in environments with different pollution levels, so they are appropriate models for environmental ecotoxicology studies (Prado et al., 2014, 2011). However, exposure to estrogenic compounds in the early stages of life tends to increase the abundance of females in relation to males (Denslow and Sepúlveda, 2007) as detected at sites S2 and S3. Although the occurrence of males with unaltered gonads in these impacted sites allows populations to be self-sustaining for a given time (Hamilton et al., 2014), reproductive changes and the presence of intersex in these sites can affect reproductive potential (fecundity) and the sustainability of the populations in the long term (Kidd et al., 2007).

5. Conclusions

This study demonstrates for the first time the effects of oestrogenic endocrine disruptors on the reproduction of the lambari *A. rivularis* in the upper Velhas River. Reproductive changes, such as intersex gonads, deviation of sex ratio for females, histopathology of oocytes, and variations in the hepatic levels of Vtg, Zrp, and IGF-I were found in sites with higher oestrogen concentrations. Males from impacted sites had higher values of Vtg and Zrp and some of them were intersex. We proposed that deficient yolk oocytes and over-ripening can be useful reproductive biomarkers in environmental monitoring of oestrogenic EDCs contamination. As populations of this species inhabit streams (lotic environment with successive rapids and pools), the constant release of oestrogens into the water causes chronic exposure, adversely affecting the populations of *A. rivularis*.

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Appendix A. Supplementaray data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.02.181.

References

Adeogun, A.O., Onibonoje, K., Ibor, O.R., Omiwole, R.A., Chukwuka, A.V., Ugwumba, A.O., Ugwumba, A.A.A., Arukwe, A., 2016. Endocrine-disruptor molecular responses, occurrence of intersex and gonado-histopathological changes in tilapia species from a tropical freshwater dam (Awba Dam) in Ibadan. Nigeria. Aquat. Toxicol. 174, 10–21.

Arantes, F.P., Santos, H.B., Rizzo, E., Sato, Y., Bazzoli, N., 2010. Profiles of sex steroids, fecundity, and spawning of the curimatã-pacu Prochilodus argenteus in the São Francisco River, downstream from the Três Marias Dam, Southeastern Brazil. Anim. Reprod. Sci. 118, 330–336.

Arsenault, J.T.M., Fairchild, W.L., MacLatchy, D.L., Burridge, L., Haya, K., Brown, S.B., 2004. Effects of water-borne 4-nonylphenol and 17β-estradiol exposures during parr-smolt transformation on growth and plasma IGF-I of Atlantic salmon (*Salmo salar* L.). Aquat. Toxicol. 66, 255–265.

Arukwe, A., Røe, K., 2008. Molecular and cellular detection of expression of vitellogenin and zona radiata protein in liver and skin of juvenile salmon (*Salmo salar*) exposed to nonylphenol. Cell Tissue Res. 331, 701–712.

Arukwe, A., Celius, T., Walther, B.T., Goksøyr, A., 2000. Effects of xenoestrogen treatment on zona radiata protein and vitellogenin expression in Atlantic salmon (*Salmo salar*). Aquat. Toxicol. 49, 159–170.

Atkinson, S.K., Marlatt, V.L., Kimpe, L.E., Lean, D.R.S., Trudeau, V.L., Blais, J.M., 2012. The occurrence of steroidal estrogens in south-eastern Ontario wastewater treatment plants. Sci. Total Environ. 430, 119–125.

Babin, P.J., Carnevali, O., Lubzens, E., Schneider, W.J., 2007. Molecular aspects of oocyte vitellogenesis in fish. In: Babin, P.J., Cerdà, J., Lubzens, E. (Eds.), The Fish Oocyte: From Basic Studies to Biotechnological Applications. Springer, Dordrecht, pp. 39–76.

Bahamonde, P.A., Munkittrick, K.R., Martyniuk, C.J., 2013. Intersex in teleost fish: are we distinguishing endocrine disruption from natural phenomena? Gen. Comp. Endocrinol. 192, 25–35.

Bahamonde, P.A., Tetreault, G.R., McMaster, M.E., Servos, M.R., Martyniuk, C.J., Munkittrick, K.R., 2014. Molecular signatures in rainbow darter (*Etheostoma caeruleum*) inhabiting an urbanized river reach receiving wastewater effluents. Aquat. Toxicol. 148, 211–220.

Bahamonde, P.A., Fuzzen, M.L., Bennett, C.J., Tetreault, G.R., McMaster, M.E., Servos, M.R., Martyniuk, C.J., Munkittrick, K.R., 2015. Whole organism responses and intersex severity in rainbow darter (*Etheostoma caeruleum*) following exposures to municipal wastewater in the Grand River basin, ON, Canada. Part A. Aquat. Toxicol. 159, 290–301.

Barber, L.B., Vajda, A.M., Douville, C., Norris, D.O., Writer, J.H., 2012. Fish endocrine disruption responses to a major wastewater treatment facility upgrade. Environ. Sci. Technol. 46, 2121–2131.

Berishvili, G., D'Cotta, H., Baroiller, J.F., Segner, H., Reinecke, M., 2006. Differential expression of IGF-I mRNA and peptide in the male and female gonad during early development of a bony fish, the tilapia *Oreochromis niloticus*. Gen. Comp. Endocrinol. 146, 204–210.

Bjerregaard, L.B., Korsgaard, B., Bjerregaard, P., 2006. Intersex in wild roach (*Rutilus rutilus*) from Danish sewage effluent-receiving streams. Ecotoxicol. Environ. Saf. 64, 321–328.

Campbell, B., 2003. Endocrine changes during onset of puberty in male spring Chinook salmon, *Oncorhynchus tshawytscha*. Biol. Reprod. 69, 2109–2117.

Carvalho, P.A., Paschoalini, A.L., Santos, G.B., Rizzo, E., Bazzoli, N., 2009. Reproductive biology of *Astyanax fasciatus* (Pisces: Characiformes) in a reservoir in Southeastern Brazil. J. Appl. Ichthyol. 25, 306–313.

Chiang, G., Barra, R., Díaz-Jaramillo, M., Rivas, M., Bahamonde, P., Munkittrick, K.R., 2015. Estrogenicity and intersex in juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to pine/eucalyptus pulp and paper production effluent in Chile. Aquat. Toxicol. 164, 126–134.

Denslow, N., Sepúlveda, M., 2007. Ecotoxicological effects of endocrine disrupting compounds on fish reproduction. In: Babin, P.J., Cerdà, J., Lubzens, E. (Eds.), The Fish Oocyte: From Basic Studies to Biotechnological Applications. Springer, Dordrecht, pp. 255–322.

Desforges, J.P.W., Peachey, B.D.L., Sanderson, P.M., White, P.A., Blais, J.M., 2010. Plasma vitellogenin in male teleost fish from 43 rivers worldwide is correlated with upstream human population size. Environ. Pollut. 158, 3279–3284.

Douxfils, J., Robert, M., Frédéric, S., Arnaud, B., Delphine, L., Jean-Pierre, T., Patrick, K., 2007. Do sewage treatment plant discharges substantially impair fish reproduction in polluted rivers? Sci. Total Environ. 372, 497–514.

Fishelson, L., 2006. Cytomorphological alterations of the thymus, spleen, head-kidney, and liver in cardinal fish (Apogonidae, Teleostei) as bioindicators of stress. J. Morphol. 267, 57–69.

Flett, P.A., Van Der Kraak, G., Munkittrick, K.R., Leatherland, J.F., 1996. Overripening as the cause of low survival to hatch in Lake Erie coho salmon (*Oncorhynchus kisutch*) embryos. Can. J. Zool. 74, 851–857.

Folmar, L.C., Denslow, N.D., Rao, V., Chow, M., Crain, D.A., Enblom, J., Marcino, J., Guillette, L.J., 1996. Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. Environ. Health Perspect. 104, 1096–1101.

Froese, R., 2006. Cube law, condition factor and weight-length relationships: history, meta-analysis and recommendations. J. Appl. Ichthyol. 22, 241–253.

Grzesiak, M., Knapczyk-Stwora, K., Ciereszko, R.E., Wieciech, I., Slomczynska, M., 2014. Alterations in luteal production of androstenedione, testosterone, and estrone, but not estradiol, during mid- and late pregnancy in pigs: effects of androgen deficiency. Theriogenology 82, 720–733.

Hamilton, P.B., Nicol, E., De-Bastos, E.S.R., Williams, R.J., Sumpter, J.P., Jobling, S., Stevens, J.R., Tyler, C.R., 2014. Populations of a cyprinid fish are self-sustaining despite widespread feminization of males. BMC Biol. 12, 1.

Harris, C.A., Hamilton, P.B., Runnalls, T.J., Vinciotti, V., Henshaw, A., Hodgson, D., Coe, T.S., Jobling, S., Tyler, C.R., Sumpter, J.P., 2011. The consequences of feminization in breeding groups of wild fish. Environ. Health Perspect. 119, 306–311.

Holbech, H., Kinnberg, K., Petersen, G.I., Jackson, P., Hylland, K., Norrgren, L., Bjerregaard, P., 2006. Detection of endocrine disrupters: evaluation of a Fish Sexual Development Test (FSDT). Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 144, 57–66.

IBGE, 2014. Sinopse do Censo e Resultados Preliminares do Universo [WWW Document]. URL. http://www.ibge.gov.br/home/ (accessed 6.21.16).

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. 104, 8897–8901.

Kinnison, M.T., Unwin, M.J., Jara, F., 2000. Macroscopic intersexuality in salmonid fishes. New Zeal. J. Mar. Freshw. Res. 34, 125–134.

Knapen, D., De Wolf, H., Knaepkens, G., Bervoets, L., Eens, M., Blust, R., Verheyen, E., 2009. Historical metal pollution in natural gudgeon populations: inferences from allozyme, microsatellite and condition factor analysis. Aquat. Toxicol. 95, 17–26.

Lima, F.C.T., Malabarba, L.R., Buckup, P.A., Pezzi da Silva, J.F., Vari, R.P., Harold, A., Benine, R., Oyakawa, O.T., Pavanelli, C.S., Menezes, N.A., Lucena, C.A.S., Malabarba, M.C.S.L., Lucena, Z.M.S., Reis, R.E., Langeani, F., Moreira, C., 2003. Genera Incertae Sedis in Characidae. In: Reis, R.E., Kullander, S.O., Ferraris-Jr, C.J. (Eds.), Check List of the Freshwater Fishes of South and Central America. EDIPUCRS, Porto Alegre, pp. 106–168.

Lintelmann, J., Katayama, A., Kurihara, N., Shore, L., Wenzel, A., 2003. Endocrine disruptors in the environment (IUPAC technical report). Pure Appl. Chem. 75, 631–681.

Lütken, C.F., 1875. Velhas-Flodens fiske. Et bidragtil Brasiliens ichthyologi. Elfter Professor J. Reinhardt indsamlinger og optegnelser. KonDank Vidensk Selsk Skrift (Kjoebenhavn) 12, 122–252.

Luzio, A., Monteiro, S.M., Garcia-Santos, S., Rocha, E., Fontaínhas-Fernandes, A.A., Coimbra, A.M., 2015. Zebrafish sex differentiation and gonad development after exposure to 17α-ethinylestradiol, fadrozole and their binary mixture: a stereological study. Aquat. Toxicol. 166, 83–95.

Luzio, A., Monteiro, S.M., Rocha, E., Fontaínhas-Fernandes, A.A., Coimbra, A.M., 2016. Development and recovery of histopathological alterations in the gonads of zebrafish (*Danio rerio*) after single and combined exposure to endocrine disruptors (17α-ethinylestradiol and fadrozole). Aquat. Toxicol. 175, 90–105.

Miura, T., Miura, C., Ohta, T., Nader, M.R., Todo, T., Yamauchi, K., 1999. Estradiol-17beta stimulates the renewal of spermatogonial stem cells in males. Biochem. Biophys. Res. Commun. 264, 230–234.

Morais, R.D.V.S., Thomé, R.G., Lemos, F.S., Bazzoli, N., Rizzo, E., 2012. Autophagy and apoptosis interplay during follicular atresia in fish ovary: A morphological and immunocytochemical study. Cell Tissue Res. 347, 467–478.

Moreira, M., Aquino, S., Coutrim, M., Silva, J., Afonso, R., 2011. Determination of endocrine disrupting compounds in waters from Rio das Velhas, Brazil, by liquid chromatography/high resolution mass spectrometry (ESI-LC-IT-TOF/MS). Environ. Technol. 32, 1409–1417.

Myers, N., Mittermeier, R.A., Mittermeier, C.G., da Fonseca, G.A., Kent, J., 2000. Biodiversity hotspots for conservation priorities. Nature 403, 853–858.

Nie, M., Yang, Y., Liu, M., Yan, C., Shi, H., Dong, W., Zhou, J.L., 2014. Environmental estrogens in a drinking water reservoir area in Shanghai: occurrence, colloidal contribution and risk assessment. Sci. Total Environ. 487, 785–791.

Noaksson, E., Linderoth, M., Gustavsson, B., Zebühr, Y., Balk, L., 2005. Reproductive status in female perch (*Perca fluviatilis*) outside a sewage treatment plant processing leachate from a refuse dump. Sci. Total Environ. 340, 97–112.

Pompeu, P.S., Alves, C.B.M., Callisto, M., 2005. The effects of urbanization on biodiversity and water quality in the Rio das Velhas Basin, Brazil. Am. Fish. Soc. Symp. 47, 11–22.

Prado, P.S., Souza, C.C., Bazzoli, N., Rizzo, E., 2011. Reproductive disruption in lambari *Astyanax fasciatus* from a Southeastern Brazilian reservoir. Ecotoxicol. Environ. Saf. 74, 1879–1887.

Prado, P.S., Pinheiro, A.P.B., Bazzoli, N., Rizzo, E., 2014. Reproductive biomarkers responses induced by xenoestrogens in the characid fish *Astyanax fasciatus* inhabiting a South American reservoir: an integrated field and laboratory approach. Environ. Res. 131, 165–173.

Randak, T., Zlabek, V., Pulkrabova, J., Kolarova, J., Kroupova, H., Siroka, Z., Velisek, J., Svobodova, Z., Hajslova, J., 2009. Effects of pollution on chub in the River Elbe, Czech Republic. Ecotoxicol. Environ. Saf. 72, 737–746.

Reinecke, M., 2010. Insulin-like growth factors and fish reproduction. Biol. Reprod. 82, 656–661.

Santos, H.B., Thomé, R.G., Arantes, F.P., Sato, Y., Bazzoli, N., Rizzo, E., 2008. Ovarian follicular atresia is mediated by heterophagy, autophagy, and apoptosis in *Prochilodus argenteus* and *Leporinus taeniatus* (Teleostei: Characiformes). Theriogenology 70: 1449–1460. http://dx.doi.org/10.1016/j.theriogenology.2008.06.091.

Schultz, M.M., Minarik, T.A., Martinovic-Weigelt, D., Curran, E.M., Bartell, S.E., Schoenfuss, H.L., 2013. Environmental estrogens in an urban aquatic ecosystem: II. Biological effects. Environ. Int. 61, 138–149.

Shved, N., Berishvili, G., Baroiller, J.F., Segner, H., Reinecke, M., 2008. Environmentally relevant concentrations of 17α-ethinylestradiol (EE2) interfere with the growth hormone (GH)/insulin-like growth factor (IGF)-I system in developing bony fish. Toxicol. Sci. 106, 93–102.

Silva, P., Rocha, M.J., Cruzeiro, C., Malhão, F., Reis, B., Urbatzka, R., Monteiro, R.A.F., Rocha, E., 2012. Testing the effects of ethinylestradiol and of an environmentally relevant mixture of xenoestrogens as found in the Douro River (Portugal) on the maturation of fish gonads—a stereological study using the zebrafish (*Danio rerio*) as model. Aquat. Toxicol. 124–125, 1–10.

Sim, W.J., Lee, J.W., Shin, S.K., Song, K.B., Oh, J.E., 2011. Assessment of fates of estrogens in wastewater and sludge from various types of wastewater treatment plants. Chemosphere 82, 1448–1453.

Tyler, C.R., Jobling, S., 2008. Roach, sex, and gender-bending chemicals: the feminization of wild fish in English rivers. Bioscience 58, 1051–1059.

Veloso-Júnior, V.C., Guimarães-Cruz, R.J., Barros, M.D.M., Barata, R.S.L., Santos, J.E., 2009. Reproduction of the lambari *Astyanax scabripinnis* (Jenyns, 1842) (Pisces: Characidae) in a small stream in Southeastern Brazil. J. Appl. Ichthyol. 25, 314–320.

Venâncio, L.P.R., Domingos, C.R., 2014. Effects of pollutants on condition index. In: de Almeida, E.A., Ribeiro, C.A.O. (Eds.), Pollution and Fish Health in Tropical Ecosystems. Taylor & Francis, London, p. 394.

Vicq, R., Matschullat, J., Leite, M.G.P., Nalini Junior, H.A., Mendonça, F.P.C., 2015. Iron Quadrangle stream sediments. Brazil: geochemical maps and reference values. Environ. Earth Sci. 74, 4407–4417.

Vieira, F., Gomes, J.P., Maia, B.P., Silva, L.G., 2015. Peixes do Quadrilátero Ferrífero - Guia de Identificação. Fundação Biodiversitas, Belo Horizonte.

4.2 Artigo 2

(Submetido para na Environmental Science and Pollution Research)

Environmental Science and Pollution Research

Stage-specific testicular protein levels of the oestrogen receptor (ERα and ERβ) and Cyp19 and association with oestrogenic contamination in the lambari Astyanax rivularis (Pisces: Characidae).

--Manuscript Draft--

Manuscript Number:	ESPR-D-18-04038R1
Full Title:	Stage-specific testicular protein levels of the oestrogen receptor (ERα and ERβ) and Cyp19 and association with oestrogenic contamination in the lambari Astyanax rivularis (Pisces: Characidae).
Article Type:	Research Article
Corresponding Author:	Elizete Rizzo Universidade Federal de Minas Gerais Belo Horizonte, MG BRAZIL
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Funding Information:	Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG 01/2015 - CVZ-APQ-00898-15) — Dr Elizete Rizzo Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq 14/2012) — Dr Elizete Rizzo
Abstract:	Oestrogens participate in various biological processes such as oogenesis, vitellogenesis, and testicular development, but studies regarding the distribution and protein levels of oestrogen receptors (ERα and ERβ) and aromatase (Cyp19) in testis are rarely investigated in fish species. The aim of the present study was to analyse the expression pattern of ERα, ERβ and Cyp19 in testis of Astyanax rivularis and, in addition, to verify if oestrogenic contamination interferes in the expression levels of these proteins. Quarterly field samplings were carried out during a reproductive cycle in a stream of the Upper Velhas River with a good conservation status (site S1). In the gonadal maturation peak (June), when ripe stage was most abundant, fish collection was made in three streams: S1, reference site, and S2 and S3, sites contaminated by untreated sewage. The results of immunohistochemistry demonstrated labelling of Cyp19 in Leydig cells and acidophilic granulocytes, but spermatogonia, Sertoli cells, spermatids and spermatozoa were also labelled. ERα was more widely distributed than ERβ being found in all developmental germ cells phases. On the other hand, ERβ was found only in spermatogonia and spermatocytes. During testicular maturation, ELISA levels for Cyp19, ERα and ERβ followed the gonadosomatic index (GSI) with significant higher values in the ripe stage. Regarding to endocrine disruption, the males

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	exposed to domestic sewage presented significant higher expression of Cyp19 and ERα when compared to the non-exposed fish. Together, our results demonstrate expression patterns of Cyp19, ERα and ERβ in the testis of A. rivularis. In addition, we indicate ERα and Cyp19 as sensitive biomarkers for monitoring of oestrogenic contamination in freshwater environments.
Response to Reviewers:	Editorial office of Environmental Science and Pollution Research Aug 28th 2018 Dear Editor Dr. Cinta Porte, We are resubmitting the manuscript "Stage-specific testicular protein levels of the oestrogen receptor (ERα and ERβ) and Cyp19 and association with oestrogenic contamination in the lambari Astyanax rivularis (Pisces: Characidae)" to publication in Environmental Science and Pollution Research. We changed the name of the manuscript at the suggestion of one of the reviewers. We would like to thank the valuable suggestions and comments that the editors and reviewers have made for the improvement of the manuscript. In order to answer the requested suggestions we made several structural modifications in the text of the manuscript and added one more figure and a table. We believe that with the requested modifications the manuscript is appropriate for publication in Environmental Science and Pollution Research. The answers to the reviewers follow below. Best regards Elizete Rizzo Cell Biology Graduate Program Department of Morphology, Institute of Biological Science Federal University of Minas Gerais, Brazil Reviewer #1: Testicular expression profile of the oestrogen receptor (ERα and ERβ) and Cyp19 in the lambari Astyanax rivularis (Pisces: Characidae) from sites with different levels of oestrogenic contamination, Brazil. The research group studied the levels profile of ERα, ERβ and Cyp19 in wild male Astyanax rivularis from different developmental stages. They combine 2 major techniques, immunohistochemistry to localize those proteins in the different cells from the testis and ELISA to quantify the levels of those proteins. The introduction was very informative, and the methodology straightforward. However, the results do not have the order associated with the methodology. Furthermore, the title states the investigation as the levels of ERα, ERβ and Cyp19 in males with different levels of oestrogenic contamination. However, the work is more than that, only one figure shows what the title state for. Answer: We agree with the reviewer that the title is not appropriate for the manuscript. We have modified it to make it more appropriate. Major comments: The group insist with the sentence of expression profile. Expression profile is associated with mRNA, not with proteins levels. It needs to be correct on the whole text. Answer: We have changed this term in all sentences. The manuscript does not have a correct organization. Answer: We reorganized the manuscript. The figure legends are incorrect.

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Answer: We rewrite the legends of the figures.

Specific comments:

Line 105: organize the effects of EDCs according to the biological level of organization from higher to lower.
Answer: Ok. Done.
Line 121: not clear the fishing sampling. The abstract state that only S1 was fish quarterly to establish reproductive fish status and S2 and S3 only during ripe stage. The methodology says all the sited were fish quarterly.
Answer: The only site that had four collection was the reference (S1) to perform the study of proteins throughout the different stages of testicular maturation. For comparison between sites was compared only by the June collection. It has been modified in the text for better understanding.
There is information about where the sites localization (GPS point), dates, etc. Usually, a table help with this information.
Answer: We put a table (Table 1) characterizing the sampling sites.

Line 125: it is not expression. It is protein levels
Answer: Done.

Line 128. Why you didn't obtain condition factor (K = BW/TL3*100)? It is a good indicator of fish body condition.
Answer: It was added in text in material and methods in lines 132-134 and in results in lines 205-206.

To keep an organization, I will change the order of the methodology in the same way the results are present.
Answer: We reorganized the manuscript.

Line 135. How much tissue do you homogenized?
Answer: 0.1g per sample. We put in text. Line 171.

Figure S1- The standard curves are not lineal. That is a basic chemistry rule. The dynamic range is not state, either the detection limits.
Answer: This exact non-linearity may be associated with calibration of the pipette. However, all dilutions followed a gradual reduction of absorbance. Regarding the amplitude and limit of detection was added in the text. This information was added in text in lines 184-190.
S2- what does it mean each column from the western blot?
Answer: We did western blotting with random samples, just to confirm specificity of antibodies.

Line 177: Organized the statistics in a way of the results are going to be shown.
Answer: Ok. Done.

Line 184. The colleagues have not report condition factor as a more reliable measurement to compare the reference site versus the polluted sites.
Answer: It was added in text in lines 205-206.

Line 185. Is it a statistical difference between site S2 and S3?
Answer: No. There is no difference between biometric parameters among fishes from S2 and S3.

Line 485. There is no Z (staining spermatozoa) at the figure.
Answer: Done. It’s indicated in figure 3d.

Line 192. Delete "weak" word
Answer: Ok. Done.

Line 493. The is no Z at the legend, but it is at the figure 2E
Answer: Ok. Done.

Line 495. What is IT?

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Answer: It’s a interstitial tissue. We added in the caption.

Line 202. GSI is figure 4A
Answer: Done.

Explain the results at the same order than the figure
Answer: Ok. Done.

Line 204. ERb is minor at two stages statistically (resting and ripening). ERa is statistically minor at 3 stages. Wrong interpretation of the stats.
Answer: It has been added in the text.

Line 207. It is incorrect to state "expression" for protein levels.
Answer: Ok. It was modified.

Line 508. Legend of figure missed the gonadosomatic index.
In addition, I suggest to show this figure as a boxplot instead of bar chart.
Answer: Ok. Done.

Line 220. What happens with the testis development between December to march?
Answer: In December the testis of this species are at resting and in March most of the individuals are in ripening.

Line 222. Protein levels, not expression.
Answer: Ok. Done.

Line 222. The example cited for rainbow trout and zebrafish, is it mRNA or protein?
Answer: In these studies mRNA was used.

Line 229-232. It seems to me that belongs more to methodology than the discussion.
Answer: Ok. You're right. We changed to methodology.

Line 259. If it is protein, is not expression.
Answer: Done.

Line 268. If it is protein, is not expression.
Answer: Done.

Line 270. If it is protein, is not expression.
Answer: Done.

Line 285. What rainbow trout tissue used to evaluate ER expression?
Answer: mRNA.

Reviewer #2: General comment
The manuscript ESPR-D-18-04038 (Testicular expression profile of the oestrogen receptor (ERα and ERβ) and Cyp19 in the lambari Astyanax rivularis (Pisces: Characidae) from sites with different levels of oestrogenic contamination, Brazil) analyzed the expression of the ERa, ERβ and Cyp19 in testes of A. rivularis and the effect of environmentally relevant concentrations of oestrogenic compounds from Upper Velhas River. The results underline the immunolocalization of ERa, ERβ and Cyp19 throughout testicular maturation and its potential use as biomarker for biomonitoring of oestrogenic contamination. However, some points should be better clarified, such as the relationship between oestrogen concentrations (ng/L), physical and chemical parameters of water and expression of the ERa, ERβ and Cyp19. A multivariate statistical analysis, such as the principal component analysis (PCA), could be added in order to analyze the relationship between all measured parameters. I recommend organizing all the text (abstract, introduction, materials and methods, results, discussion, conclusion) and figures in a single logical order ("ERa, ERβ and Cyp19"), such as described in the title. Some additional concerns are detailed. I recommend the publication of this manuscript after minor revision.

Specific comments
Abstract

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Line 39-40. "…the distribution and expression of aromatase (Cyp19) and oestrogen receptors (ERα and ERβ) in testis are…" = "…the distribution and expression of oestrogen receptors (ERα and ERβ) and aromatase (Cyp19) in testis are…"
Answer: Ok. Done.

Line 59. Change the keyword "oestrogen receptors" (similar to the title);
Answer: Ok. We change to oestrogenic pathway. Line 59.

Line 59. "Neotropical, fish" = "neotropical fish"
Answer: Ok. Done. Line 59.

Introduction
Line 80-85. Describe the ER expression in fish.
Answer: Added in text between lines 81-86.

Line 104. "Astyanax rivularis" = "Astyanax rivularis (Lutken, 1875)".
Answer: Yes is the same.

Line 108; 111. "Astyanax rivularis (Lutken, 1875)" = "A. rivularis"; ""Astyanax rivularis" = "A. rivularis".
Answer: Ok. Done.

Materials and methods
Line 123-124. Add the water quality parameters in Table S1 or in supplementary material. Describe the differences in the physical and chemical parameters of water in the results, as well as its effects on biomarker response.
Answer: OK. Done. Lines 206-207.

Line 125-126; 133-134. Describe the number of fish per site (n = 10 per site?).
Answer: It was added in text. Lines 128-130 and 147.

Line 126. "mg.L-1" = "mg L-1"
Answer: Done.

Line 178-181. All data presented normal distribution? What was the statistical test applied to non-parametric data?
Answer: We used Kruskal-Wallis for non-parametric data. We added in the text. Line 195.

Results
Add the physical and chemical parameters of water and the biometric parameters (TL, BW, GW and GSI) in the supplementary material. What the effect of oestrogen concentrations (ng/L), physical and chemical parameters of water on biometric parameters?
Answer: It was added. Oestrogenic compounds can affect the production of thyroid hormones (T3 and T4) leading to a reduction in the growth of contaminated fish. We added a discussion on biometric parameters in lines 245-249.

Line 201-202. The relation between the expression pattern and GSI is not clear. Add correlation and regression analysis (statistic analysis).
A multivariate statistical analysis, such as the principal component analysis (PCA), could be add in order to analyse the relationship between physical and chemical parameters, oestrogen concentrations and all measured biomarkers.
Answer: Correlation between GSI and protein levels was added in the results, lines 222-224.
We did multivariate analysis, however we chose canonical correspondence analysis (CCA) for making an association between the oestrogenic compounds and the chosen biomarkers. Lines 199-200 and 233-236 and figure 8.

Discussion
The relationship between environmentally relevant concentrations of oestrogens and its bioaccumulation and biological effects should be better clarified and discussed.
Answer: We discuss better the biological effects that endocrine disrupters cause. Lines 245-249, 307-308, 312-313 and 315-316.

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	Line 263-264. Add statistic analysis in the results. Answer: We added throughout the results Reviewer #3: The paper by Weber et al was designed to study role of estrogens on various biological processes such as the distribution and expression of aromatase (Cyp19) and estrogen receptors (ERα and ERβ) in the testis of Astyanax rivularis and to verify if estrogenic contamination interferes in the expression levels of these proteins from their ambient environment. Samples were collected from the Upper Velhas River with a good conservation status and other contaminated sites by untreated sewage. They basically applied immunochemical techniques showing the immunological labelling of Cyp19 in Leydig cells and acidophilic granulocytes, spermatogonia, Sertoli cells, spermatids and spermatozoa were also labelled. They also showed that ERα was more widely distributed than ERβ being found in all developmental germ cells phases. They concluded that ERα and Cyp19 may be sensitive biomarkers for monitoring of estrogenic contamination in freshwater environments. The study was mostly well designed and have a regional significance. The analytical protocols were also appropriate with a reasonable level of validation, although - it was very narrow. The biggest problem with the paper was that the authors drew a BIG line on biomarker development from a very narrow analytical approach. The discussion is mostly a repetition of the result and not focused and failed to put the data presented in the paper in a broader context, instead a rather awkward focus was placed on irrelevant issues about development of germ cells. Answer: The objective of this study is to determine the expression of the three proteins analyzed along the reproductive cycle of males of a fish species, which is a large gap in the literature in this regard. Thus, this study is the first to address the expression of these molecules at the protein level. In addition, we use these proteins as possible biomarkers of endocrine disruption because they are affected by exposure to oestrogenic compounds. Thus the discussion followed this trend of discussing the expression of these proteins in different cell types throughout the reproductive cycle and then discussing with other papers how these proteins are regulated when animals are exposed to oestrogenic endocrine disrupters. We have added more information in the discussion to demonstrate how the up-regulation of these proteins causes reproductive effects. Other comments 1.Page 2, Line 54. Please change sensible to sensitive Answer: Ok. Done. Now is line 55. 2.I will suggest the ELISA and western blot validation assay be moved from the SI to the main manuscript. These informations are very critical to the validity of the data and should not be hidden in the SI. Consider including a subtitle "Validation of immunochemal assays" Answer: We agree and thank you for the suggestion. We added in lines 184-190 in material and methods but we prefer to leave the figures in supplementary material because it is not a direct result. 3.Page 3, please delete line 82-85 "In addition, …………. (…….., Thomas 2012). Irrelevant to the study. Answer: Ok. Done. 4.Page 4, line 114, please "EDCs………fish" which is factually incorrect. Answer: Ok. It was modified.
Additional Information:	
Question	**Response**
§Are you submitting to a Special Issue?	No

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Editorial office of Environmental Science and Pollution Research

Aug 28th 2018

Dear Editor Dr. Cinta Porte,

We are resubmitting the manuscript "**Stage-specific testicular protein levels of the oestrogen receptor (ERα and ERβ) and Cyp19 and association with oestrogenic contamination in the lambari *Astyanax rivularis* (Pisces: Characidae)**" to publication in Environmental Science and Pollution Research. We changed the name of the manuscript at the suggestion of one of the reviewers.

We would like to thank the valuable suggestions and comments that the editors and reviewers have made for the improvement of the manuscript. In order to answer the requested suggestions we made several structural modifications in the text of the manuscript and added one more figure and a table. We believe that with the requested modifications the manuscript is appropriate for publication in Environmental Science and Pollution Research. The answers to the reviewers follow below.

Best regards

Elizete Rizzo
Cell Biology Graduate Program
Department of Morphology, Institute of Biological Science
Federal University of Minas Gerais, Brazil

Reviewer #1: Testicular expression profile of the oestrogen receptor (ERα and ERβ) and Cyp19 in the lambari *Astyanax rivularis* (Pisces: Characidae) from sites with different levels of oestrogenic contamination, Brazil.

The research group studied the levels profile of ERα, ERβ and Cyp19 in wild male *Astyanax rivularis* from different developmental stages. They combine 2 major techniques, immunohistochemistry to localize those proteins in the different cells from the testis and ELISA to quantify the levels of those proteins.

The introduction was very informative, and the methodology straightforward. However, the results do not have the order associated with the methodology. Furthermore, the title states the investigation as the levels of ERα, ERβ and Cyp19 in males with different levels of oestrogenic contamination. However, the work is more than that, only one figure shows what the title state for.

Answer: We agree with the reviewer that the title is not appropriate for the manuscript. We have modified it to make it more appropriate.

Major comments:

The group insist with the sentence of expression profile. Expression profile is associated with mRNA, not with proteins levels. It needs to be correct on the whole text.

Answer: We have changed this term in all sentences.

The manuscript does not have a correct organization.

Answer: We reorganized the manuscript.

The figure legends are incorrect.

Answer: We rewrite the legends of the figures.

Specific comments:

Line 105: organize the effects of EDCs according to the biological level of organization from higher to lower.

Answer: Ok. Done.

Line 121: not clear the fishing sampling. The abstract state that only S1 was fish quarterly to establish reproductive fish status and S2 and S3 only during ripe stage. The methodology says all the sited were fish quarterly.

Answer: The only site that had four collection was the reference (S1) to perform the study of proteins throughout the different stages of testicular maturation. For comparison between sites was compared only by the June collection. It has been modified in the text for better understanding.

There is information about where the sites localization (GPS point), dates, etc. Usually, a table help with this information.

Answer: We put a table (Table 1) characterizing the sampling sites.

Line 125: it is not expression. It is protein levels

Answer: Done.

Line 128. Why you didn't obtain condition factor (K = BW/TL3*100)? It is a good indicator of fish body condition.

Answer: It was added in text in material and methods in lines 132-134 and in results in lines 205-206.

To keep an organization, I will change the order of the methodology in the same way the results are present.

Answer: We reorganized the manuscript.

Line 135. How much tissue do you homogenized?

Answer: 0.1g per sample. We put in text. Line 171.

Figure S1- The standard curves are not lineal. That is a basic chemistry rule. The dynamic range is not state, either the detection limits.

Answer: This exact non-linearity may be associated with calibration of the pipette. However, all dilutions followed a gradual reduction of absorbance. Regarding the

amplitude and limit of detection was added in the text. This information was added in text in lines 184-190.

S2- what does it mean each column from the western blot?

Answer: We did western blotting with random samples, just to confirm specificity of antibodies.

Line 177: Organized the statistics in a way of the results are going to be shown.

Answer: Ok. Done.

Line 184. The colleagues have not report condition factor as a more reliable measurement to compare the reference site versus the polluted sites.

Answer: It was added in text in lines 205-206.

Line 185. Is it a statistical difference between site S2 and S3?

Answer: No. There is no difference between biometric parameters among fishes from S2 and S3.

Line 485. There is no Z (staining spermatozoa) at the figure.

Answer: Done. It's indicated in figure 3d.

Line 192. Delete "weak" word

Answer: Ok. Done.

Line 493. The is no Z at the legend, but it is at the figure 2E

Answer: Ok. Done.

Line 495. What is IT?

Answer: It's a interstitial tissue. We added in the caption.

Line 202. GSI is figure 4A

Answer: Done.

Explain the results at the same order than the figure

Answer: Ok. Done.

Line 204. ERb is minor at two stages statistically (resting and ripening). ERa is statistically minor at 3 stages. Wrong interpretation of the stats.

Answer: It has been added in the text.

Line 207. It is incorrect to state "expression" for protein levels.

Answer: Ok. It was modified.

Line 508. Legend of figure missed the gonadosomatic index.

In addition, I suggest to show this figure as a boxplot instead of bar chart.

Answer: Ok. Done.

Line 220. What happens with the testis development between December to march?

Answer: In December the testis of this species are at resting and in March most of the individuals are in ripening.

Line 222. Protein levels, not expression.

Answer: Ok. Done.

Line 222. The example cited for rainbow trout and zebrafish, is it mRNA or protein?

Answer: In these studies mRNA was used.

Line 229-232. It seems to me that belongs more to methodology than the discussion.

Answer: Ok. You're right. We changed to methodology.

Line 259. If it is protein, is not expression.

Answer: Done.

Line 268. If it is protein, is not expression.

Answer: Done.

Line 270. If it is protein, is not expression.

Answer: Done.

Line 285. What rainbow trout tissue used to evaluate ER expression?

Answer: mRNA.

Reviewer #2: General comment

The manuscript ESPR-D-18-04038 (Testicular expression profile of the oestrogen receptor (ERα and ERβ) and Cyp19 in the lambari *Astyanax rivularis* (Pisces: Characidae) from sites with different levels of oestrogenic contamination, Brazil) analyzed the expression of the ERa, ERβ and Cyp19 in testes of A. rivularis and the effect of environmentally relevant concentrations of oestrogenic compounds from Upper Velhas River. The results underline the immunolocalization of ERa, ERβ and Cyp19 throughout testicular maturation and its potential use as biomarker for biomonitoring of oestrogenic contamination. However, some points should be better clarified, such as the relationship between oestrogen concentrations (ng/L), physical and chemical parameters of water and expression of the ERa, ERβ and Cyp19. A multivariate statistical analysis, such as the principal component analysis (PCA), could be added in order to analyze the relationship between all measured parameters. I recommend organizing all the text (abstract, introduction, materials and methods, results, discussion, conclusion) and figures in a single logical order ("ERa, ERβ and Cyp19"), such as described in the title. Some additional concerns are detailed. I recommend the publication of this manuscript after minor revision.

Specific comments

Abstract

Line 39-40. "…the distribution and expression of aromatase (Cyp19) and oestrogen receptors (ERα and ERβ) in testis are…" = "…the distribution and expression of oestrogen receptors (ERα and ERβ) and aromatase (Cyp19) in testis are…"

Answer: Ok. Done.

Line 59. Change the keyword "oestrogen receptors" (similar to the title);

Answer: Ok. We change to oestrogenic pathway. Line 59.

Line 59. "Neotropical, fish" = "neotropical fish"

Answer: Ok. Done. Line 59.

Introduction

Line 80-85. Describe the ER expression in fish.

Answer: Added in text between lines 81-86.

Line 104. "Astyanax rivularis" = "Astyanax rivularis (Lutken, 1875)".

Answer: Yes is the same.

Line 108; 111. "Astyanax rivularis (Lutken, 1875)" = "A. rivularis"; ""Astyanax rivularis" = "A. rivularis".

Answer: Ok. Done.

Materials and methods

Line 123-124. Add the water quality parameters in Table S1 or in supplementary material. Describe the differences in the physical and chemical parameters of water in the results, as well as its effects on biomarker response.

Answer: OK. Done. Lines 206-207.

Line 125-126; 133-134. Describe the number of fish per site (n = 10 per site?).

Answer: It was added in text. Lines 128-130 and 147.

Line 126. "mg.L-1" = "mg L-1"

Answer: Done.

Line 178-181. All data presented normal distribution? What was the statistical test applied to non-parametric data?

Answer: We used Kruskal-Wallis for non-parametric data. We added in the text. Line 195.

Results

Add the physical and chemical parameters of water and the biometric parameters (TL, BW, GW and GSI) in the supplementary material. What the effect of oestrogen concentrations (ng/L), physical and chemical parameters of water on biometric parameters?

Answer: It was added. Oestrogenic compounds can affect the production of thyroid hormones (T3 and T4) leading to a reduction in the growth of contaminated fish. We added a discussion on biometric parameters in lines 245-249.

Line 201-202. The relation between the expression pattern and GSI is not clear. Add correlation and regression analysis (statistic analysis).

A multivariate statistical analysis, such as the principal component analysis (PCA), could be add in order to analyse the relationship between physical and chemical parameters, oestrogen concentrations and all measured biomarkers.

Answer: Correlation between GSI and protein levels was added in the results, lines 222-224.

We did multivariate analysis, however we chose canonical correspondence analysis (CCA) for making an association between the oestrogenic compounds and the chosen biomarkers. Lines 199-200 and 233-236 and figure 8.

Discussion

The relationship between environmentally relevant concentrations of oestrogens and its bioaccumulation and biological effects should be better clarified and discussed.

Answer: We discuss better the biological effects that endocrine disrupters cause. Lines 245-249, 307-308, 312-313 and 315-316.

Line 263-264. Add statistic analysis in the results.

Answer: We added throughout the results

Reviewer #3:

The paper by Weber et al was designed to study role of estrogens on various biological processes such as the distribution and expression of aromatase (Cyp19) and estrogen receptors (ERα and ERβ) in the testis of *Astyanax rivularis* and to verify if estrogenic contamination interferes in the expression levels of these proteins from their ambient environment. Samples were collected from the Upper Velhas River with a good conservation status and other contaminated sites by untreated sewage. They basically applied immunochemical techniques showing the immunological labelling of Cyp19 in Leydig cells and acidophilic granulocytes, spermatogonia, Sertoli cells, spermatids and spermatozoa were also labelled. They also showed that ERα was more widely distributed than ERβ being found in all developmental germ cells phases. They concluded that ERα and Cyp19 may be sensitive biomarkers for monitoring of estrogenic contamination in freshwater environments. The study was mostly well designed and have a regional significance. The analytical protocols were also appropriate with a reasonable level of validation, although - it was very narrow. The biggest problem with the paper was that the authors drew a BIG line on biomarker development from a very narrow analytical approach. The discussion is mostly a repetition of the result and not focused and failed to put the data presented in the paper in a broader context, instead a rather awkward focus was placed on irrelevant issues about development of germ cells.

Answer: The objective of this study is to determine the expression of the three proteins analyzed along the reproductive cycle of males of a fish species, which is a large gap in the literature in this regard. Thus, this study is the first to address the expression of these molecules at the protein level. In addition, we use these proteins as possible biomarkers of endocrine disruption because they are affected by exposure to oestrogenic compounds. Thus the discussion followed this trend of discussing the expression of these proteins in different cell types throughout the reproductive cycle and then discussing with other papers how these proteins are regulated when animals are exposed to oestrogenic endocrine disrupters. We have added more information in the discussion to demonstrate how the up-regulation of these proteins causes reproductive effects.

Other comments

1. Page 2, Line 54. Please change sensible to sensitive

Answer: Ok. Done. Now is line 55.

2. I will suggest the ELISA and western blot validation assay be moved from the SI to the main manuscript. These informations are very critical to the validity of the data and should not be hidden in the SI. Consider including a subtitle "Validation of immunochemal assays"

Answer: We agree and thank you for the suggestion. We added in lines 184-190 in material and methods but we prefer to leave the figures in supplementary material because it is not a direct result.

3. Page 3, please delete line 82-85 "In addition, …………. (…….., Thomas 2012). Irrelevant to the study.

Answer: Ok. Done.

4. Page 4, line 114, please "EDCs………fish" which is factually incorrect.

Answer: Ok. It was modified.

Manuscript

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Stage-specific testicular protein levels of the oestrogen receptor (ERα and ERβ) and Cyp19 and association with oestrogenic contamination in the lambari *Astyanax rivularis* (Pisces: Characidae).

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Acknowledgments

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Abstract

Oestrogens participate in various biological processes such as oogenesis, vitellogenesis, and testicular development, but studies regarding the distribution and protein levels of oestrogen receptors (ERα and ERβ) and aromatase (Cyp19) in testis are rarely investigated in fish species. The aim of the present study was to analyse the expression pattern of ERα, ERβ and Cyp19 in testis of *Astyanax rivularis* and, in addition, to verify if oestrogenic contamination interferes in the expression levels of these proteins. Quarterly field samplings were carried out during a reproductive cycle in a stream of the Upper Velhas River with a good conservation status (site S1). In the gonadal maturation peak (June), when ripe stage was most abundant, fish collection was made in three streams: S1, reference site, and S2 and S3, sites contaminated by untreated sewage. The results of immunohistochemistry demonstrated labelling of Cyp19 in Leydig cells and acidophilic granulocytes, but spermatogonia, Sertoli cells, spermatids and spermatozoa were also labelled. ERα was more widely distributed than ERβ being found in all developmental germ cells phases. On the other hand, ERβ was found only in spermatogonia and spermatocytes. During testicular maturation, ELISA levels for Cyp19, ERα and ERβ followed the gonadosomatic index (GSI) with significant higher values in the ripe stage. Regarding to endocrine disruption, the males exposed to domestic sewage presented significant higher expression of Cyp19 and ERα when compared to the non-exposed fish. Together, our results demonstrate expression patterns of Cyp19, ERα and ERβ in the testis of *A. rivularis.* In addition, we indicate ERα and Cyp19 as sensitive biomarkers for monitoring of oestrogenic contamination in freshwater environments.

- **Keywords:** testis, oestrogenic pathway, aromatase, neotropical fish, endocrine disruption, pollution.

1. Introduction

Oestrogens participate in various biological processes in males and females, such as oogenesis, vitellogenesis, and spermatogonial self-renewal (Gustafsson 2003; Hess 2003; Nelson and Habibi 2013). In adult males, oestrogen biosynthesis occurs mainly in Leydig cells in the testis, but aromatase is also detected in developing germ cells and spermatozoa (Carreau and Hess 2010; Carreau et al. 2011). Fertility of males depends on the process of aromatization of androgens into oestrogen by the enzyme P450 aromatase (Cyp19) (Carreau et al. 2011). The effects oestrogens are mediated by intracellular oestrogen receptors (ERα and ERβ) that belong to a superfamily of nuclear receptors (Nelson and Habibi 2013). When oestrogens molecules are absent, ERs are found in a complex associated with chaperones (HSPs) in the cytoplasm (Nelson and Habibi 2013). However, in the presence of the ligand these complexes are disassembled. After that, ERs binds to oestrogen molecule, migrates to the nucleus and acts as a transcription factor in specific regions of the DNA, called oestrogen responsive elements (EREs) (Björnström and Sjöberg 2005; Thomas 2012).

In mammals, ERβ is widely distributed throughout the male reproductive system and express in different germ cells developmental phases but ERα is highly specific, being found in Leydig cells of the adult testis and epithelium of the efferent ductules (Carreau and Hess, 2010). Few studies demonstrate the expression of ER types in different male germ cells in fish. Viñas and Piferrer (2008) demonstrated that ERα and ERβ are expressed in spermatogonia and spermatocytes, however only the ERα is expressed in spermatids and spermatozoa. In relation to the interstitial cells the levels of expression are not yet known.

The release of raw domestic sewage into rivers and streams promotes the entry of several anthropogenic substances, several of which are able to modulate the endocrine system of aquatic organisms, including oestrogenic endocrine disrupters-chemicals (EDCs) (Stevens et al. 2003; Lishman et al. 2006). Oestrogenic EDCs act as oestrogen receptors agonists and may be more potent than the natural hormone itself (Denslow and Sepúlveda 2007). Some studies have demonstrated an up-regulation in the expression of oestrogen receptors, especially ERα, in fish exposed to oestrogenic compounds (Kloas et al. 2000; Wen et al. 2013; Bahamonde et al. 2014). In addition to oestrogen receptors, an increase in Cyp19 is also detected in environments with domestic and industrial sewage discharge and, aromatase levels can be higher in males than females in environments with high contamination levels (Ibor et al. 2016). Studies also demonstrate that some endocrine disrupters may also interfere in the non-genomic steroids pathway,

including membrane progestin receptors (mPRs) and GPR30 (Das and Thomas 1999; Thomas and Doughty 2004; Ropero et al. 2006).

The Velhas River basin is located in the central region of Minas Gerais, south-eastern Brazil, with its upper region located between two hotspot ecosystems, Atlantic Rainforest and Cerrado biomes (Myers et al. 2000). With 761 km of extension, the Velhas River is the largest tributary of the São Francisco River, flowing into right bank of the upper São Francisco River. The intense urbanization and industrial activity coupled with insufficient sewage treatment make the Velhas River, one of the most polluted rivers of Minas Gerais state (Veado et al. 2000; Alves and Pompeu 2005; Moreira et al. 2011). Recent study carried out in the upper Velhas River showed endocrine disruption in the *Astyanax rivularis*, with presence of reduction in body size, intersex fish, changes in spermatogenesis and increase in hepatic levels of vitellogenin (Vtg), zona radiata proteins (Zrp) and reduction of insulin-like growth factor (IGF-I) (Weber et al. 2017).

The fish species *Astyanax rivularis* (Lutken, 1875) belongs to Characidae family, popularly known as lambari, has small body size and inhabits streams formed by rapids and substrate composed of rocks from Upper Velhas River (Lima et al. 2003). This species has an omnivorous feeding habit, consuming various food items (Veloso-Júnior et al. 2009). *Astyanax rivularis* is abundant in environments with different degrees of pollution, so it becomes an appropriate species for studies of aquatic contamination (Weber et al. 2017).

Considering that there are no protein-level studies that analyze ERs and aromatase in fish and how these proteins are regulated in environments contaminated with oestrogenic compounds, the aim of the present study was to analyse the expression of ERα, ERβ and Cyp19 in testis of *A. rivularis* and, in addition, to verify if oestrogenic contamination in upper Velhas River interferes in the expression levels of these proteins.

2. Material and methods

2.1. Fish sampling

Specimens of *A. rivularis* were captured in three streams of the upper Velhas River: quarterly in S1, reference site, with a pristine status, and only one sampling (june) in S2 and S3, contaminated sites that receive municipal domestic sewage of cities and districts (Table 1). The water quality parameters and

oestrogenic contamination of each sampling site were reported at Weber et al. (2017), and a summarize in Table S1.

To assess the testicular protein levels of ERα, ERβ and Cyp19 throughout the year, we used 10 males per sampling (40 fishes) in site S1 and to compare among sites (S1, S2 and S3) we use 10 fish per site (30 fishes). Alive fish were euthanized with immersion in Eugenol 85 mgL^{-1}. Biometry was performed for all fish and total length (TL), body weight (BW) and gonadal weight (GW) were obtained from each individual and gonadosomatic index (GSI = 100 GW/BW) was calculated for each fish. The Fulton condition factor ($K = 100\ BW/TL^3$) was calculated to analyze the health condition of the animals among sampling sites. All collection methods were approved by the Brazilian standards of animal experimentation (Weber et al. 2017).

2.2. Histology

For analyses of the testicular maturation, 10 testis samples of each maturity stage from S1 site were fixed in Bouin's fluid for 8 to 12 h at room temperature and then kept in 70% ethanol. The samples were dehydrated in ethanol, embedded in paraffin, sectioned at 5 µm thickness, and stained with haematoxylin-eosin (HE). Based on the distribution of germ cells four stages of gonadal maturation were used: (1) resting, (2) ripening, (3) ripe, and (4) spent (Carvalho et al. 2009). A total of 10 specimens of each maturity stage from site S1 was random chosen for immunohistochemistry of ERα, ERβ and Cyp19, during gonadal maturation.

2.3. Immunohistochemistry

Testis samples (n=10 per sampling) from reference site (S1) male fish were submitted to immunofluorescence and immunoperoxidase, as previously described by Morais et al. (2016), using the primaries antibodies, rabbit anti-human ERα (MC-20, sc-542), rabbit anti-human ERβ (H-150, sc-8974) and rabbit anti-human Cyp19 (H-300, sc-30086) (Santa Cruz Biotechnology, Inc. USA). The antibodies used in the present study are produced in mammals because no antibodies for fish are available. However, all epitopes of the antibodies correspond to the C-terminal region, which is the most conserved among species (Nelson and Habibi 2013). The specificity of the antibodies was confirmed by Western blotting.

The primary antibody was omitted in one slide for negative control. The sections were submitted in sodium citrate buffer pH 6.0, at 95 ºC for 30 min for antigen retrieval and exposure of specific epitopes.

Bovine serum albumin (BSA) at 2% was used to block non-specific binding. After that, sections were incubated in a humid camera overnight at 4 °C with the primary antibodies described above and used at 1:50 dilution. The sections were then incubated with anti-rabbit immunoglobulin G (IgG) secondary antibody conjugated with Alexa fluor 488 at 1:200, and nuclear staining was made with 40,6-diamidino-2-phenylindole (DAPI) at 1:500. The images were analysed in a Fluorescence Microscope Axio Imager Z2-ApoTome 2 Zeiss. To confirm the cellular distribution of the proteins, serial sections were also submitted to immunoperoxidase, following similar procedures used for the immunofluorescence. Endogenous peroxidase was blocked with 3% hydrogen peroxide before incubation with primary antibody. The biotinylated secondary antibody (Dako EnVision™ + Dual Link System-HRP) was used for 30 min. The 3’3-diaminobenzidine tetrahydrochloride hydrate (DAB) was used for immunohistochemical reactions, and testis sections were counterstained with haematoxylin.

2.4. ELISA Assays and Western Blotting

During fish collection, a total of 30 testis samples at ripe stage from S1, S2 and S3 were kept in liquid nitrogen and then kept in a freezer at -80 °C. The samples were subjected to indirect ELISA assay, as previously described by Prado et al. (2014). Briefly, the frozen samples (0.1g tissue per sample) were homogenised in extraction buffer Tris-HCl with aprotinin and phenylmethylsulfonyl, centrifuged at 15,000 g for 60 min at 4 °C, and supernatants were stored at -80 °C. Total protein was determined by the Bradford method using bovine serum albumin (BSA) as standard. Duplicate samples of 100 µg/ml were incubated for one hour in ELISA microplates (Nunc, Denmark), blocked non-specific bindings with 2% BSA, and incubated at 37 °C for one hour with same primary antibodies used at immunohistochemistry at 1:200 dilution (Santa Cruz Biotechnology, Inc. USA). After that, the microplates were incubated with secondary antibody conjugated anti-IgG with peroxidase (Sigma, St. Louis, MO) for one hour at 37 °C at 1:500. The reaction was revealed with 200 µl of o-phenylenediamine dihydrochloride (OPD) (Sigma, St. Louis, MO, USA) and hydrogen peroxide. The microplates were read at 492 nm using a BioTek Instruments Inc. spectrophotometer. For validation of the ELISA assays, dilution curves of testis homogenates were performed for each protein analysed and the specificity of each antibody were determined by Western blotting using protocol established by Thomé et al. (2012).

The validation of assays was confirmed by western blotting analysis and it was possible to determine a band of approximately 55 kDa for Cyp19, ERα of 68 kDa, ERβ of 54 kDa (Fig. S1). These molecular

1 186 weights are in agreement with those previously described for these three proteins in different vertebrate
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3 187 species (Zhou et al. 2002; Oliveira et al. 2011; Oliveira et al. 2012; Caneguim et al. 2013). The dilution
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5 188 curves of the three proteins analyzed demonstrated a gradual reduction of proteins from the more
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7 189 concentrated to the less concentrated (Fig. S2). The dynamic range for all proteins was 0 to 4.5 OD. The
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9 190 absorbances of all samples were within this range.
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13 192 **2.5. Statistical analyses**

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15 193 GraphPad 5.0 software Inc., version 3.05, San Diego, CA, USA was used in the statistical analyses.
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17 194 One-way analysis of variance followed by Tukey's test was used to compare fish total length, body
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19 195 weight, K and Kruskal-Wallis followed by Dunn's test to compare GSI, and absorbance of ERα, ERβ and
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21 196 Cyp19 among gonadal maturation stages and sites. To analyze the correlation between protein levels and
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23 197 GSI throughout the year Pearson's correlation was used. Data were expressed as mean ± standard error
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25 198 and considered significant at $p < 0.05$.
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27 199 A canonical correspondence analysis (CCA) was performed between concentrations of oestrogenic
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29 200 compounds and biomarkers among sites (Table S1). This analysis was performed in PAST 3.2 program.
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33 202 **3. Results**

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35 203 Fish from S1 showed body size from 7.8 to 11.4 cm TL and 7.03 to 22 g BW. Comparing sites, ripe
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37 204 males from S1 presented higher values of TL and BW in relation to males from sites S2 and S3 (F=6.65,
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39 205 p = 0.0075 and F=8.16, p = 0.003, respectively) (Table S1). The Fulton condition (K) of S2 males had
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41 206 lower values than those observed in the S1 and S3 sites (F=5.72, p=0.01). All estrogenic compounds had
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43 207 higher concentration values at sites S2 and S3 when compared to S1 (Table S1).
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48 209 **3.2. Testicular protein levels of ERα, ERβ and CYP19**

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50 210 Regarding ERα, strong staining was observed in spermatogonia in resting stage (Fig. 3a, b). In
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52 211 ripening and ripe stages, an intense staining was observed in Leydig cells, Sertoli cells, spermatogonia
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54 212 and spermatocytes, while spermatids and spermatozoa were slightly labelled (Fig. 1c-e). In spent stage,
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56 213 spermatogonia were also labelled (Fig. 1f). Immunolabelling for ERβ was localised in spermatogonia (Fig
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58 214 2a), in Leydig cells, Sertoli cells, and spermatocytes during ripening and ripe stages (Fig. 2b-d). In spent
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60 215 stage, marking on spermatogonia was detected (Fig. 2e). Immunohistochemistry evidenced the
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distribution of the labelling in the testis. Labelling for Cyp19 occurred in interstitial cells and acidophilic granulocytes in the resting stage (Fig 3a, b). On the other hand, Sertoli cells, Leydig cells, spermatids and spermatozoa showed strong staining while spermatogonia, spermatocytes presented a reaction at ripening and ripe stages (Fig 3c-e). In spent stage, only some connective cells and spermatogonia were immunolabelled by Cyp19.

The testicular expression levels of ERα, ERβ and Cyp19 during gonadal maturation was analysed by ELISA in specimens from S1, a site with few anthropogenic interference (Fig.4a). The correlation between GSI and ERα was positive but no significative (r = 0.17, p=0.38) but ERβ (r = 0.64, p=0.0004) and CYP19 (r = 0.51, p=0.007) were positive and significant. Significantly minor expression occurred at ripening for ERα (H=8.21, p = 0.0418), resting and ripening for ERβ (H=21.50, $p < 0.0001$), and resting, ripening and spent stages for Cyp19 (H=19.27, p = 0.0002) (Fig 4b, c and d, respectively).

3.3. Testicular protein levels variations of ERα, ERβ and CYP19 among sites

Comparing testicular expression levels among sampling sites by ELISA (Fig. 5), higher values of ERα (H=19.45, $p < 0.0001$) and Cyp19 (H=28.73, $p < 0.0001$) were found at S2 and S3, contaminated sites by oestrogenic EDCs when compared with S1, reference site. No statistical difference was observed for GSI (H=1.36, p = 0.51) and testicular levels of ERβ among sampling sites (H=0.80, p = 0.66).

The first axis of the CCA counted 98% of the variation of concentrations of contaminants in the water and biomarkers analyzed. All estrogenic compounds were directly related to the S2 and S3 sites and also the biomarkers ERα and CYP19. On the other hand, TL and BW had greater relationship with S1 males (Figure 6).

4. Discussion

The lambaris of the genus *Astyanax* are widely distributed in South American river basins, and are frequently found in environments with different pollution levels, so they has been used as bioindicators in ecotoxicology studies (Prado et al. 2011; Paulino et al. 2014; Prado et al. 2014; Tolussi et al. 2018). In the headwaters of the Velhas River, *A. rivularis* reproduces in streams, spawn in September, and its populations are self-sustainable despite the endocrine disruption detected in the streams contaminated by domestic sewage (Weber et al. 2017).

Lower values of total length and body weight were observed in males at sites contaminated in the present study. This pattern is common in studies of estrogenic endocrine disruption in both field and laboratory approaches (Randak et al. 2009; Silva et al. 2012; Prado et al. 2014; Bahamonde et al. 2015; Ibor et al. 2016). Some estrogenic compounds promotes the reduction of thyroid hormones like T3 and T4, affecting the growth of individuals in the population (Naderi et al. 2014).

Testicular maturation of *A. rivularis* occurs throughout the year, with a peak of ripening males in March, ripe in June, while spent and resting stages are predominant in September and December, respectively (Weber et al. 2017), periods when the specimens of present study were caught in order to address the protein levels of ERα, ERβ and Cyp19 in the testis. In this regard, the mRNA expression patterns and distribution of Cyp19 and ERs was reported in the testis of few fish species as rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*) (Hinfray et al. 2013; Delalande et al. 2015). In addition, the available studies focus only on one subtype of ER, mainly ERα and did not address the gonadal maturation stage when these proteins were analysed (Nelson and Habibi 2013). Still, most studies evaluated mRNA for ERs rather than protein levels, thus comparisons between different fish species can be an arduous task and not always feasible.

In testis, ERα and ERβ levels is highly variable in vertebrates and exhibit a species-specific expression pattern (Carreau and Hess 2010). In mammals, it was believed that ERα was found in some cells such as Leydig cells, myoid peritubular cells, Sertoli cells and only some germ cells (Kotula-Balak et al. 2005; Lucas et al. 2008). However, studies with several species of fish demonstrated the presence of ERα mRNA in all developing germ cells stages: spermatogonia, spermatocytes, spermatids and spermatozoa (He et al. 2003; Viñas and Piferrer 2008; Zhu et al. 2008), similar to results obtained in the present study.

In *A. rivularis*, protein levels of ERβ presented a greater specificity of labelling than the ERα, with labelling for ERβ being observed on Sertoli cells, Leydig cells, spermatogonia and spermatocytes. Similar results were found in sea bass (*Dicentrarchus labrax*), that evidenced ERα mRNA expression in all germ line cells while ERβ mRNA was expressed only in spermatogonia and spermatocytes (Viñas and Piferrer 2008). Interestingly, the protein levels of both ERα and ERβ followed the gonadal maturation in *A. rivularis*, suggesting that the two forms of nuclear ER has similar patterns, with a peak during ripe stage. Experimental studies in rainbow trout and Atlantic cod (*Gadus morhua*) also showed mRNA expression

1 274 of the two receptors increasing along gonadal maturation (Nagasawa et al. 2014; Delalande et al. 2015), thus supporting the findings of the present study.

In the present study, the labelling by Cyp19 in acidophilic granulocytes indicates that these cells can convert testosterone into oestrogen in *A. rivularis*. In the gilthead seabream *Sparus aurata*, experimental studies show that E2 promotes a mobilization of acidophilic granulocytes into the testis in the post-spawning stage period, demonstrating that these cells play an important role in the beginning of the new cycle of spermatogenesis (Liarte et al. 2007; Liarte et al. 2011). Furthermore, a recent study reported that fish leukocytes express *Cyp19* genes (Szwejser et al. 2017), thus corroborating a role for acidophilic granulocytes in the conversion androgen to oestrogen in fish testis. Indeed, the endocrine system modulates immune system through circulating hormones, including oestrogens (Burgos-Aceves et al. 2016; Szwejser et al. 2017).

In the ripe testis of *A. rivularis*, Cyp19 labelling was observed in Leydig cells, Sertoli cells, spermatogonia, spermatids and spermatozoa as also reported in mouse and humans (Lambard et al. 2004). These data suggest that oestrogens can participate in spermatogonia self-renewal in the spermatogonial phase (Miura et al. 1999; Schulz et al. 2010) and also plays a role in spermiogenesis (Delalande et al. 2015). Oestrogens stimulate the Sertoli cell to express platelet-derived endothelial cells growth factor (PDECGF), thereby stimulating the self-renewal of spermatogonia (Miura et al. 2003). In humans and monkeys, labelling for Cyp19 was found in the spermatozoa midpiece, suggesting a possible role of oestrogen in the energy production for sperm motility (Aquila et al. 2004; Solakidi et al. 2005). In the rodent, *Myodes glareolus*, testicular Cyp19 is more abundant during the breeding season (Bilińska et al. 2001), these data being in agreement with results of the present study, where the higher protein levels of Cyp19 was obtained at the ripe stage. In the Velhas River, breeding season peak for *A. rivularis* occurs in June (Weber et al. 2017), dry period when ripe males were sampled in this study.

The highest protein levels of ERα and Cyp19 at the impacted sites of the present study are associated with endocrine disruption in the Velhas River, since that males of *A. rivularis* inhabiting S2 and S3 showed a significant increase in the protein levels of ERα and Cyp19, coinciding with an higher Vtg hepatic protein levels (Weber et al. 2017). Indeed, environmental oestrogens induce up-regulation of ERα, and induction of Vtg mRNA expression in fish males (Wen et al. 2013). On the other hand, Vtg induction can have only a little effect on the mRNA expression of hepatic ERβ (Lee Pow et al. 2016). Studies achieved in zebrafish and goldfish (*Carassius auratus*) indicate that ERβ subtypes may play a

supporting role in Vtg induction through up-regulation of ERα (Griffin et al. 2013). A field study showed that contamination by oestrogenic EDCs such as polychlorinated biphenyl (PCBs) and organochlorine pesticides (OCPs) promote an increase in hepatic Cyp19 mRNA of two fish species in Nigerian rivers, *Tilapia guinensis* and *Sarotherodon galileaus* and may deregulate several processes, such as formation of intersex males, testicular degeneration and aromatization of testosterone (Ibor et al. 2016). Furthermore, experimental administration of E2 on zebrafish males induced an increase in Cyp19 mRNA in the testis, but, no significant difference was observed with the control group (Hinfray et al. 2013). In mammalian testis, the administration of atrazine, an estrogenic disruptor found in herbicides, also promoted an increase in Cyp19 levels in Wistar rats after 40 days of exposure, can cause morphological and physiological damage in the testis (Martins-Santos et al. 2017).

In support to our results, a study carried out with *Gambusia affinis* males in China also found higher expression of hepatic ERα mRNA in contaminated rivers by environmental oestrogens, promoting a development of the female-like hemal fin, thus demonstrating a feminization of males (Wen et al. 2013). In rainbow trout exposed experimentally to 17β-oestradiol, similar results to the present study were found with increased mRNA ERα and no increase for mRNA ERβ (Benninghoff and Williams 2008; Esterhuyse et al. 2010). Another experimental study carried out with juveniles of *Rutilus rutilus* have shown that the administration of environmentally relevant concentrations of 17α-ethinylestradiol (EE2) increases the body levels of ERα, ERβ and Cyp19 mRNA, promoting the feminization of fish males (Lange et al. 2008). On the other hand, field studies with *Etheostoma caeruleum* and *Astyanax fasciatus* did not detect an increase of ERα mRNA in males contaminated by domestic sewage (Bahamonde et al. 2014; Tolussi et al. 2018). These differences indicate that xenobiotics which cause endocrine disruption through binding to nuclear receptors (ERs) in the genomic pathway, can also interfere with steroid actions mediated through membrane receptors including GPR30 (Ropero et al. 2006; Thomas and Dong 2006). Therefore, it is important investigate the non-classical steroid actions, non-genomic pathway, when investigating action pathways of environmental contaminants (Thomas 2012).

In summary, our results demonstrate for first time the immunolocalisation of ERα, ERβ and Cyp19 throughout testicular maturation in a Neotropical fish species. In addition, we indicate ERα and Cyp19 as sensible biomarkers for monitoring of oestrogenic contamination in freshwater environments. These results demonstrate the need to implement management programs in order to conservation the fish populations in the headwaters of the Velhas River.

References

Alves CBM, Pompeu PS (2005) Historical Changes in the Rio das Velhas Fish Fauna—Brazil. Am Fish Soc Symp 45:587–602.

Aquila S, Sisci D, Gentile M, et al (2004) Estrogen receptor (ER)α and ERβ are both expressed in human ejaculated spermatozoa: Evidence of their direct interaction with phosphatidylinositol-3-OH kinase/Akt pathway. J Clin Endocrinol Metab 89:1443–1451. doi: 10.1210/jc.2003-031681

Bahamonde PA, Fuzzen ML, Bennett CJ, et al (2015) Whole organism responses and intersex severity in rainbow darter (Etheostoma caeruleum) following exposures to municipal wastewater in the Grand River basin, ON, Canada. Part A. Aquat Toxicol 159:290–301.

Bahamonde PA, Tetreault GR, McMaster ME, et al (2014) Molecular signatures in rainbow darter (Etheostoma caeruleum) inhabiting an urbanized river reach receiving wastewater effluents. Aquat Toxicol 148:211–220. doi: 10.1016/j.aquatox.2014.01.010

Benninghoff AD, Williams DE (2008) Identification of a transcriptional fingerprint of estrogen exposure in rainbow trout liver. Toxicol Sci 101:65–80. doi: 10.1093/toxsci/kfm238

Bilińska B, Schmalz-Frczek B, Kotula M, Carreau S (2001) Photoperiod-dependent capability of androgen aromatization and the role of estrogens in the bank vole testis visualized by means of immunohistochemistry. Mol Cell Endocrinol 178:189–198. doi: 10.1016/S0303-7207(01)00427-0

Björnström L, Sjöberg M (2005) Mechanisms of Estrogen Receptor Signaling: Convergence of Genomic and Nongenomic Actions on Target Genes. Mol Endocrinol 19:833–842. doi: 10.1210/me.2004-0486

Burgos-Aceves MA, Cohen A, Smith Y, Faggio C (2016) Estrogen regulation of gene expression in the teleost fish immune system. Fish Shellfish Immunol 58:42–49. doi: 10.1016/j.fsi.2016.09.006

Caneguim BH, Luz JS da, Valentini SR, et al (2013) Immunoexpression of aromatase and estrogen receptors β in stem spermatogonia of bullfrogs indicates a role of estrogen in the seasonal spermatogonial mitotic activity. Gen Comp Endocrinol 182:65–72.

Carreau S, Bouraima-Lelong H, Delalande C (2011) Estrogens: new players in spermatogenesis. Reprod Biol 11:174–93. doi: 10.1016/S1642-431X(12)60065-5

Carreau S, Hess RA (2010) Oestrogens and spermatogenesis. Philos Trans R Soc Lond B Biol Sci 365:1517–35. doi: 10.1098/rstb.2009.0235

Carvalho PA, Paschoalini AL, Santos GB, et al (2009) Reproductive biology of Astyanax fasciatus (Pisces: Characiformes) in a reservoir in Southeastern Brazil. J Appl Ichthyol 25:306–313. doi: 10.1111/j.1439-0426.2009.01238.x

Das S, Thomas P (1999) Pesticides interfere with the nongenomic action of a progestogen on meiotic maturation by binding to its plasma membrane receptor on fish oocytes. Endocrinology 140:1953–1956. doi: 10.1210/endo.140.4.6781

Delalande C, Goupil AS, Lareyre JJ, Le Gac F (2015) Differential expression patterns of three aromatase genes and of four estrogen receptors genes in the testes of trout (Oncorhynchus mykiss). Mol Reprod Dev 82:694–708. doi: 10.1002/mrd.22509

Denslow N, Sepúlveda M (2007) Ecotoxicological effects of endocrine disrupting compounds on fish reproduction. In: Babin PJ, Cerdà J, Lubzens E (eds) The Fish Oocyte: From Basic Studies to Biotechnological Applications. Springer, Dordrecht, pp 255–322

Esterhuyse MM, Helbing CC, van Wyk JH (2010) Isolation and characterization of three estrogen receptor transcripts in Oreochromis mossambicus (Peters). J Steroid Biochem Mol Biol 119:26–34. doi: 10.1016/j.jsbmb.2009.12.002

Griffin LB, January KE, Ho KW, et al (2013) Morpholino-mediated knockdown of ERα, ERβa, and ERβb mRNAs in zebrafish (Danio rerio) embryos reveals differential regulation of estrogen-inducible genes. Endocrinology 154:4158–4169. doi: 10.1210/en.2013-1446

Gustafsson JÅ (2003) What pharmacologists can learn from recent advances in estrogen signalling. Trends Pharmacol Sci 24:479–485. doi: 10.1016/S0165-6147(03)00229-3

He C-L, Du J-L, Lee Y-H, et al (2003) Differential messenger RNA transcription of androgen receptor and estrogen receptor in gonad in relation to the sex change in protandrous black porgy, Acanthopagrus schlegeli. Biol Reprod 69:455–61. doi: 10.1095/biolreprod.102.015040

Hess RA (2003) Estrogen in the adult male reproductive tract: a review. Reprod Biol Endocrinol 1:52. doi: 10.1186/1477-7827-1-52

Hinfray N, Nóbrega RH, Caulier M, et al (2013) Cyp17a1 and cyp19a1 in the zebrafish testis are differentially affected by oestradiol. J Endocrinol 216:375–388. doi: 10.1530/JOE-12-0509

Ibor OR, Adeogun AO, Fagbohun OA, Arukwe A (2016) Gonado-histopathological changes, intersex and endocrine disruptor responses in relation to contaminant burden in Tilapia species from Ogun River, Nigeria. Chemosphere 164:248–262.

Kloas W, Schrag B, Ehnes C, Segner H (2000) Binding of xenobiotics to hepatic estrogen receptor and plasma sex steroid binding protein in the teleost fish, the common carp (Cyprinus carpio). Gen Comp Endocrinol 119:287–99. doi: 10.1006/gcen.2000.7521

Kotula-Balak M, Gancarczyk M, Sadowska J, Bilinskai B (2005) The expression of aromatase, estrogen receptor alpha and estrogen receptor beta in mouse Leydig cells in vitro that derived from cryptorchid males. Eur J Histochem 49:59–62.

Lambard S, Galeraud-Denis I, Saunders PTK, Carreau S (2004) Human immature germ cells and ejaculated spermatozoa contain aromatase and oestrogen receptors. J Mol Endocrinol 32:279–289. doi: 10.1677/jme.0.0320279

Lange A, Katsu Y, Ichikawa R, et al (2008) Altered sexual development in roach (Rutilus rutilus) exposed to environmental concentrations of the pharmaceutical 17α-Ethinylestradiol and associated expression dynamics of aromatases and estrogen receptors. Toxicol Sci 106:113–123. doi: 10.1093/toxsci/kfn151

Lee Pow CSD, Yost EE, Aday DD, Kullman SW (2016) Sharing the Roles: An Assessment of Japanese Medaka Estrogen Receptors in Vitellogenin Induction. Environ Sci Technol 50:8886–8895. doi: 10.1021/acs.est.6b01968

Liarte S, Chaves-Pozo E, Abellán E, et al (2011) 17β-Estradiol regulates gilthead seabream professional phagocyte responses through macrophage activation. Dev Comp Immunol 35:19–27. doi: 10.1016/j.dci.2010.07.007

Liarte S, Chaves-Pozo E, García-Alcazar A, et al (2007) Testicular involution prior to sex change in gilthead seabream is characterized by a decrease in DMRT1 gene expression and by massive leukocyte infiltration. Reprod Biol Endocrinol 5:20. doi: 10.1186/1477-7827-5-20

Lima FCT, Malabarba LR, Buckup PA, et al (2003) Genera Incertae Sedis in Characidae. In: Reis RE, Kullander SO, Ferraris-Jr CJ (eds) Check List of the Freshwater Fishes of South and Central America. EDIPUCRS, Porto Alegre, pp 106–168

Lishman L, Smyth SA, Sarafin K, et al (2006) Occurrence and reductions of pharmaceuticals and personal care products and estrogens by municipal wastewater treatment plants in Ontario, Canada. Sci Total Environ 367:544–558. doi: 10.1016/j.scitotenv.2006.03.021

Lucas TFG, Siu ER, Esteves C a, et al (2008) 17beta-estradiol induces the translocation of the estrogen receptors ESR1 and ESR2 to the cell membrane, MAPK3/1 phosphorylation and proliferation of cultured immature rat Sertoli cells. Biol Reprod 78:101–114. doi: 10.1095/biolreprod.107.063909

Martins-Santos E, Pimenta CG, Campos PRN, et al (2017) Persistent testicular structural and functional alterations after exposure of adult rats to atrazine. Reprod Toxicol. doi: 10.1016/j.reprotox.2017.08.010

Miura T, Miura C, Ohta T, et al (1999) Estradiol-17beta stimulates the renewal of spermatogonial stem cells in males. Biochem Biophys Res Commun 264:230–234. doi: 10.1006/bbrc.1999.1494

Miura T, Ohta T, Miura CI, Yamauchi K (2003) Complementary Deoxyribonucleic Acid Cloning of Spermatogonial Stem Cell Renewal Factor. Endocrinology 144:5504–5510. doi: 10.1210/en.2003-0800

Morais RDVS, Thomé RG, Santos HB, et al (2016) Relationship between bcl-2, bax, beclin-1, and cathepsin-D proteins during postovulatory follicular regression in fish ovary. Theriogenology 85:1118–1131. doi: 10.1016/j.theriogenology.2015.11.024

Moreira M, Aquino S, Coutrim M, et al (2011) Determination of endocrine disrupting compounds in waters from Rio das Velhas, Brazil, by liquid chromatography/high resolution mass spectrometry (ESI-LC-IT-TOF/MS). Environ Technol 32:1409–1417. doi: 10.1080/09593330.2010.537829

Myers N, Mittermeier RA, Mittermeier CG, et al (2000) Biodiversity hotspots for conservation priorities. Nature 403:853–858. doi: 10.1038/35002501

Naderi M, Wong MYL, Gholami F (2014) Developmental exposure of zebrafish (Danio rerio) to bisphenol-S impairs subsequent reproduction potential and hormonal balance in adults. Aquat Toxicol 148:195–203.

Nagasawa K, Presslauer C, Kirtiklis L, et al (2014) Sexually dimorphic transcription of estrogen receptors in cod gonads throughout a reproductive cycle. J Mol Endocrinol 52:357–371. doi: 10.1530/JME-13-0187

Nelson ER, Habibi HR (2013) Estrogen receptor function and regulation in fish and other vertebrates. Gen Comp Endocrinol 192:15–24. doi: 10.1016/j.ygcen.2013.03.032

Oliveira AG, Dornas RAP, Mahecha GAB, Oliveira CA (2011) Occurrence and cellular distribution of estrogen receptors ERα and ERβ in the testis and epididymal region of roosters. Gen Comp Endocrinol 170:597–603.

Oliveira RL, Nogueira JC, Mahecha GAB, Oliveira CA (2012) Seasonal variation in estrogen receptor ERα, but not ERβ, androgen receptor and aromatase, in the efferent ductules and epididymis of the big fruit-eating bat Artibeus lituratus. Gen Comp Endocrinol 179:1–13. doi: 10.1016/j.ygcen.2012.06.028

Paulino MG, Benze TP, Sadauskas-Henrique H, et al (2014) The impact of organochlorines and metals on wild fish living in a tropical hydroelectric reservoir: Bioaccumulation and histopathological biomarkers. Sci Total Environ 497–498:293–306. doi: 10.1016/j.scitotenv.2014.07.122

Prado PS, Pinheiro APB, Bazzoli N, Rizzo E (2014) Reproductive biomarkers responses induced by xenoestrogens in the characid fish Astyanax fasciatus inhabiting a South American reservoir: An integrated field and laboratory approach. Environ Res 131:165–173. doi: 10.1016/j.envres.2014.03.002

Prado PS, Souza CC, Bazzoli N, Rizzo E (2011) Reproductive disruption in lambari Astyanax fasciatus from a Southeastern Brazilian reservoir. Ecotoxicol Environ Saf 74:1879–1887. doi: 10.1016/j.ecoenv.2011.07.017

Randak T, Zlabek V, Pulkrabova J, et al (2009) Effects of pollution on chub in the River Elbe, Czech Republic. Ecotoxicol Environ Saf 72:737–746. doi: 10.1016/j.ecoenv.2008.09.020

Ropero AB, Alonso-Magdalena P, Ripoll C, et al (2006) Rapid endocrine disruption: Environmental estrogen actions triggered outside the nucleus. J Steroid Biochem Mol Biol 102:163–169. doi: 10.1016/j.jsbmb.2006.09.019

Schulz RW, França LR, Lareyre JJ, et al (2010) Spermatogenesis in fish. Gen Comp Endocrinol 165:390–411. doi: 10.1016/j.ygcen.2009.02.013

Silva P, Rocha MJ, Cruzeiro C, et al (2012) Testing the effects of ethinylestradiol and of an environmentally relevant mixture of xenoestrogens as found in the Douro River (Portugal) on the maturation of fish gonads-A stereological study using the zebrafish (Danio rerio) as model. Aquat Toxicol 124–125:1–10. doi: 10.1016/j.aquatox.2012.07.002

Solakidi S, Psarra A-MG, Nikolaropoulos S, Sekeris CE (2005) Estrogen receptors alpha and beta (ERalpha and ERbeta) and androgen receptor (AR) in human sperm: localization of ERbeta and AR in mitochondria of the midpiece. Hum Reprod 20:3481–7. doi: 10.1093/humrep/dei267

Stevens JL, Northcott GL, Stern GA, et al (2003) PAHs, PCBs, PCNs, organochlorine pesticides, synthetic musks, and polychlorinated n-alkanes in U.K. sewage sludge: Survey results and implications. Environ Sci Technol 37:462–467. doi: 10.1021/es020161y

Szwejser E, Verburg-van Kemenade BML, Maciuszek M, Chadzinska M (2017) Estrogen-dependent seasonal adaptations in the immune response of fish. Horm Behav 88:15–24. doi: 10.1016/j.yhbeh.2016.10.007

Thomas P (2012) Rapid steroid hormone actions initiated at the cell surface and the receptors that mediate them with an emphasis on recent progress in fish models. Gen Comp Endocrinol 175:367–383. doi: 10.1016/j.ygcen.2011.11.032

Thomas P, Dong J (2006) Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: A potential novel mechanism of endocrine disruption. J Steroid Biochem Mol Biol 102:175–179. doi: 10.1016/j.jsbmb.2006.09.017

Thomas P, Doughty K (2004) Disruption of rapid, nongenomic steroid actions by environmental chemicals: Interference with progestin stimulation of sperm motility in Atlantic croaker. Environ Sci Technol 38:6328–6332. doi: 10.1021/es0403662

Thomé RG, Domingos FFT, Santos HB, et al (2012) Apoptosis, cell proliferation and vitellogenesis during the folliculogenesis and follicular growth in teleost fish. Tissue Cell 44:54–62. doi: 10.1016/j.tice.2011.11.002

Tolussi CE, Gomes ADO, Kumar A, et al (2018) Environmental pollution affects molecular and biochemical responses during gonadal maturation of Astyanax fasciatus (Teleostei: Characiformes: Characidae). Ecotoxicol Environ Saf 147:926–934. doi: 10.1016/j.ecoenv.2017.09.056

Veado MARV, Oliveira AH De, Veado JCC, et al (2000) Analysis and distribution of metals in the Paraopeba and the Das Velhas Rivers, Brazil. Water SA 26:249–254.

Veloso-Júnior VC, Guimarães-Cruz RJ, Barros MDM, et al (2009) Reproduction of the lambari Astyanax scabripinnis (Jenyns, 1842) (Pisces: Characidae) in a small stream in Southeastern Brazil. J Appl Ichthyol 25:314–320. doi: 10.1111/j.1439-0426.2008.01152.x

Viñas J, Piferrer F (2008) Stage-Specific Gene Expression During Fish Spermatogenesis as Determined by Laser-Capture Microdissection and Quantitative-PCR in Sea Bass (Dicentrarchus labrax) Gonads1. Biol Reprod 79:738–747. doi: 10.1095/biolreprod.108.069708

Weber AA, Moreira DP, Melo RMC, et al (2017) Reproductive effects of oestrogenic endocrine disrupting chemicals in Astyanax rivularis inhabiting headwaters of the Velhas River, Brazil. Sci Total Environ 592:693–703. doi: 10.1016/j.scitotenv.2017.02.181

Wen R, Xie Y, Wan C, Fang Z (2013) Estrogenic and androgenic effects in mosquitofish (Gambusia affinis) from streams contaminated by municipal effluent in Guangzhou, China. Aquat Toxicol 132–133:165–172. doi: 10.1016/j.aquatox.2013.02.010

Zhou Q, Nie R, Prins GS, et al (2002) Localization of androgen and estrogen receptors in adult male mouse reproductive tract. J Androl 23:870–881. doi: 10.1002/j.1939-4640.2002.tb02345.x

Zhu P, Zhang Y, Zhuo Q, et al (2008) Discovery of four estrogen receptors and their expression profiles during testis recrudescence in male Spinibarbus denticulatus. Gen Comp Endocrinol 156:265–276. doi: 10.1016/j.ygcen.2008.01.017

Figure Captions

Fig. 1. Immunolocalisation for ERα in testis of *Astynax rivularis*. (a, c, e, f) Sections stained by immunoperoxidase, and (b, d) immunofluorescence (green) and nucleus stained by DAPI (blue). (a, b) Immunostaining of spermatogonia (G) at resting stage, (c, d, e) labelling of spermatogonia (G), spermatocytes (C), spermatids (T) in ripening, spermatozoa (Z) and ripe stages, (f) strong staining of spermatogonia (G) in spent stage. Bar scale: 2μm (a, b, f), 10 μm (c, d), 6 μm (e).

Fig. 2. Immunolocalisation for ERβ in testis of *Astynax rivularis*. (a, c) Sections stained by immunofluorescence (green) and nucleus stained by DAPI (blue), and immunoperoxidase (b, d, e). (a) Labelling of spermatogonia (G) in resting stage, (b) Staining of spermatogonia (G), spermaytocytes (C) and Leydig cells (insert) in ripening stage, (c) Staining of spermatogonia (G), spermaytocytes (C) and no staining in interstitial cells (IT), (d) Immunostaining of spermatogonia and spermatocytes mainly in ripe stage, (e) Strong immunolabelling of spermatogonia in spent stage and no staining in intertistial tissue (IT). Bar scale: 4μm (a), 8 μm (b), 2 μm (c), 18 μm (d, e).

Fig. 3. Immunolocalisation for Cyp19 in testis of *Astynax rivularis*. (a, c, d) Sections stained by immunoperoxidase, and (b, e) immunofluorescence (green) and nucleus stained by DAPI (blue). (a) Labelling of interstitial cells in resting stage, (b) Labelling of acidophilic granulocytes (arrowheads) at resting stage, (insert acidophilic granulocytes stained by haematoxylin–eosin in histological sections); (c) Weak immunostaining of spermatogonia (arrow) and immunolabelling in Leydig cells (c, insert), (d) strong staining spermatozoa (Z) in ripening stage and immunolabelling in Sertoli cells (d, insert), (e) Strong staining by immunofluorescence of spermatids and spermatozoa in ripe stage. Bar scale: 4μm (a), 8 μm (b, c), 15 μm (d, e).

Fig. 4. Relation between gonadosomatic index (GSI), and expression of aromatase (Cyp19) and oestrogen receptors (ERα and ERβ) during testicular maturation of *A. rivularis* from the upper Velhas River, south-eastern Brazil. GSI (a), and box-plot graphs of ELISA assays for Cyp19 (b), ERα (c) and ERβ (d). Data

represent absorbance values by indirect ELISA at 492 nm from 10 males/testicular maturity stage. Different letters indicate significant differences among sites ($p < 0.05$).

Fig. 5. Box-plot graphs of gonadossomatic index (a) and Testicular protein levels of (b) aromatase (Cyp19), (c) oestrogen receptor alfa (ERα), and (d) oestrogen receptor beta (ERβ) in ripe males of *A. rivularis* from three streams from the upper Vellhas River, south-eastern Brazil: (S1) reference site, (S2) and (S3) sites contaminated with domestic sewage. Data are expressed as mean ± standard error (n = 10 males/site) of indirect ELISA absorbance values at 492 nm. Different letters indicate significant differences between groups ($p < 0.05$).

Fig. 6. Canonical correspondence analysis (CCA) between the estrogenic compounds of the water and the biomarkers used.

Figure 1

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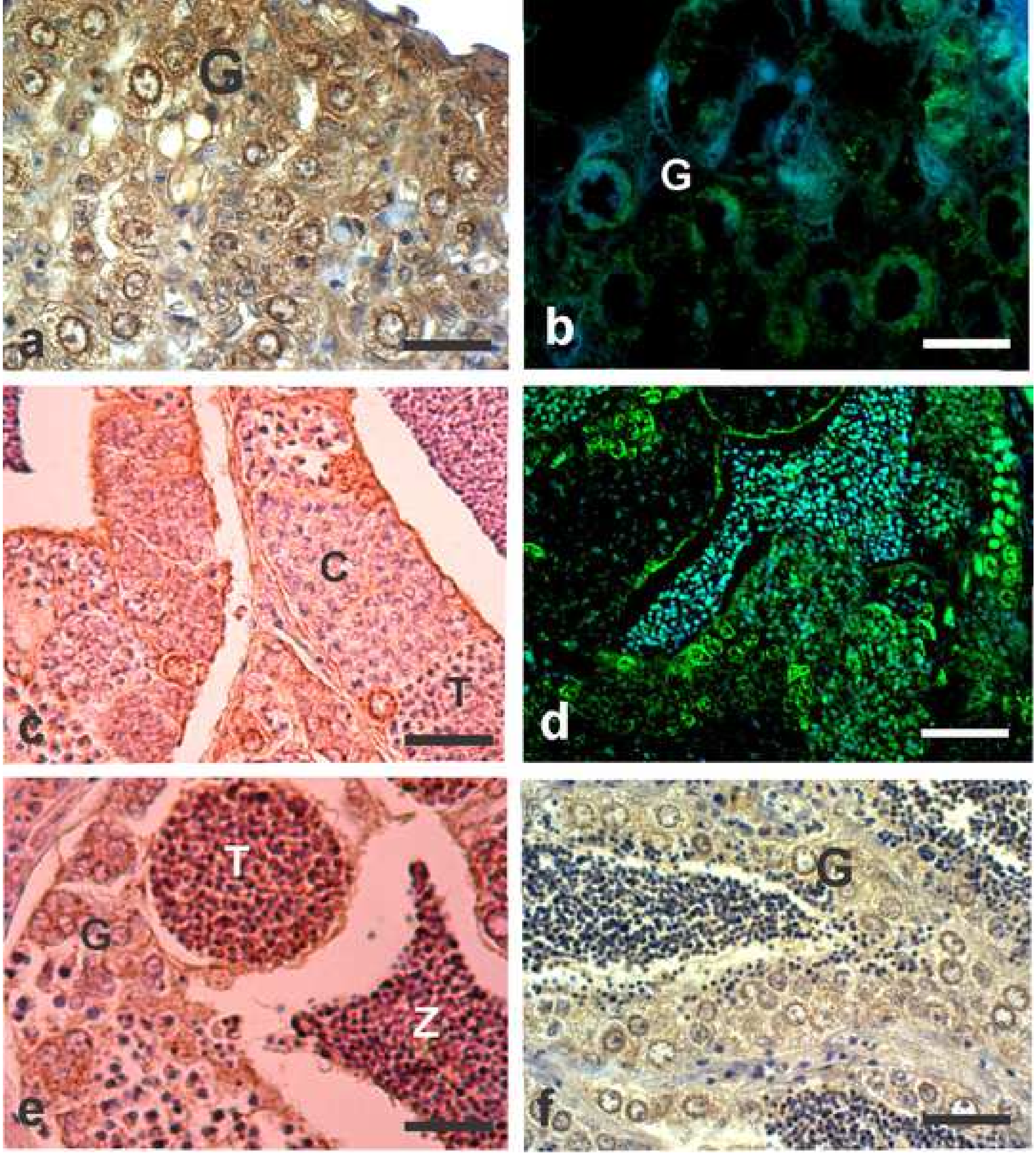

Figure 2

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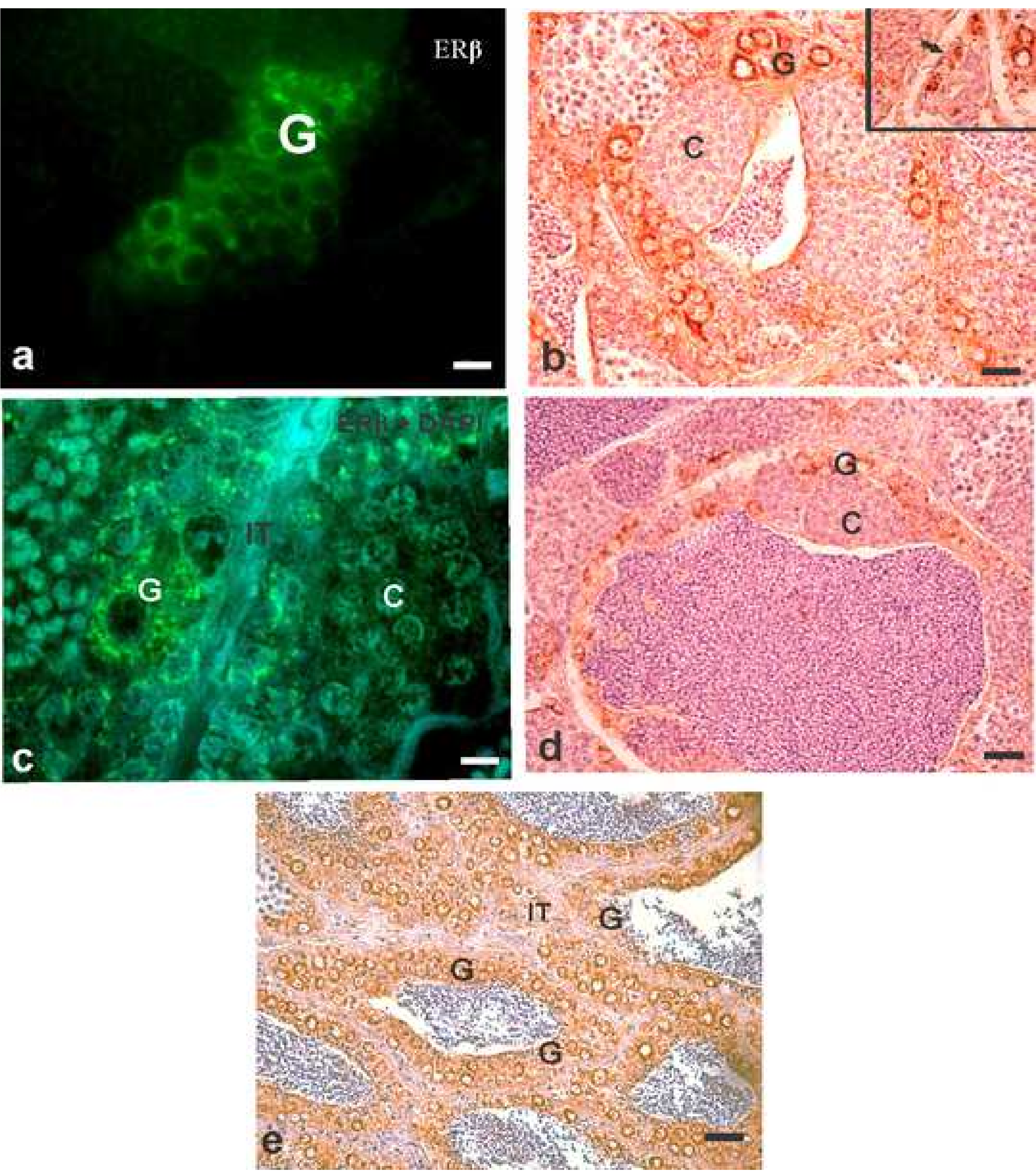

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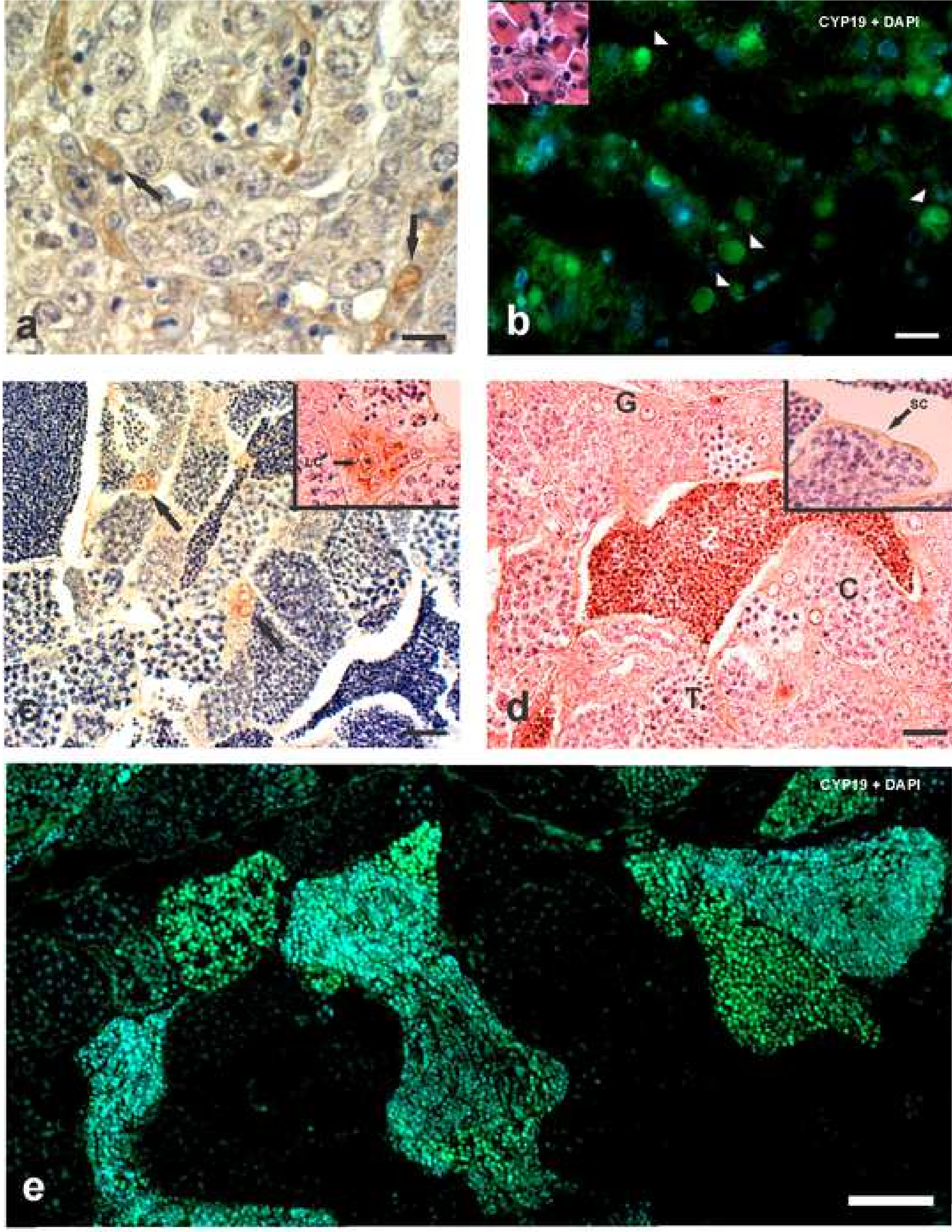

Figure 4

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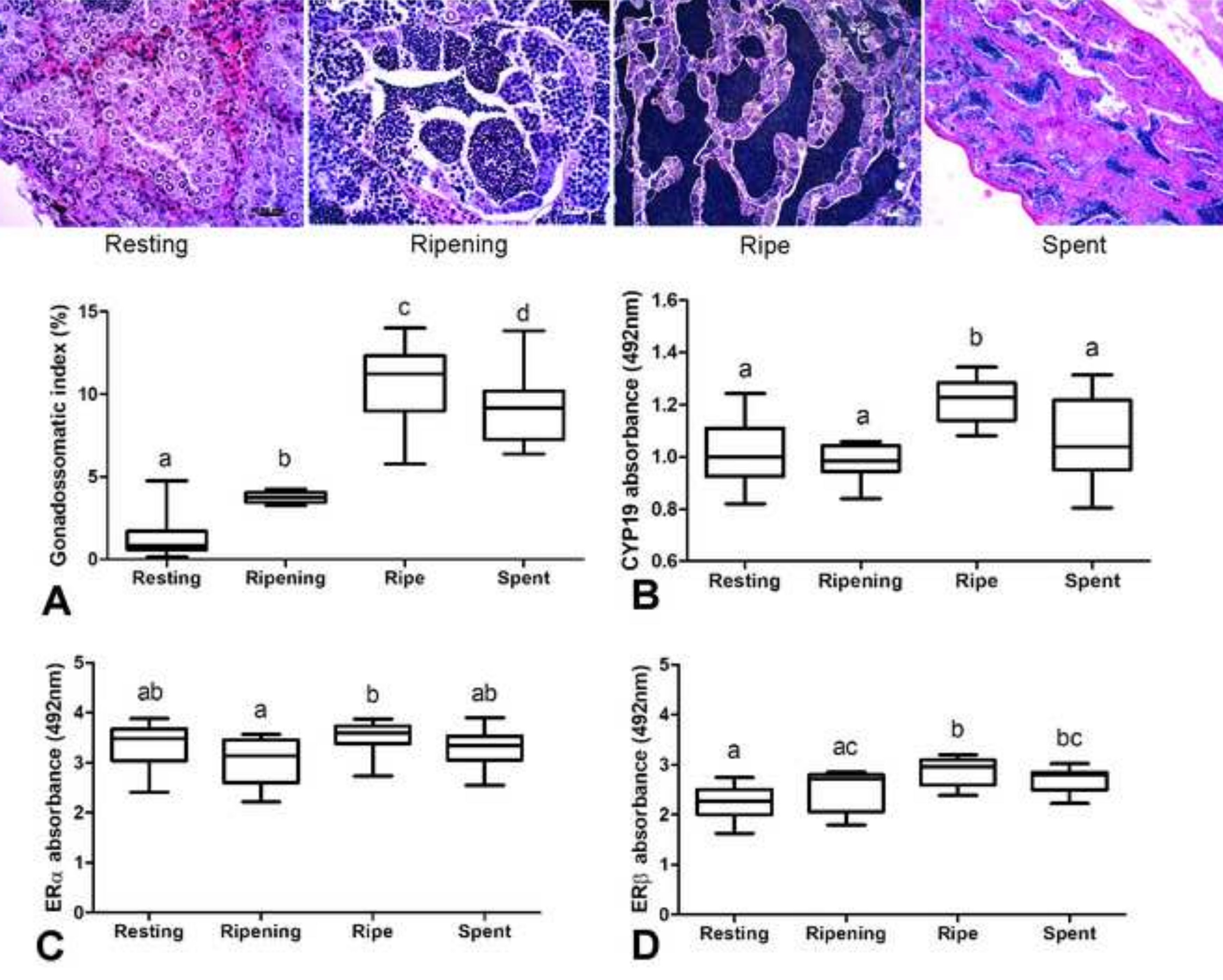

Figure 5

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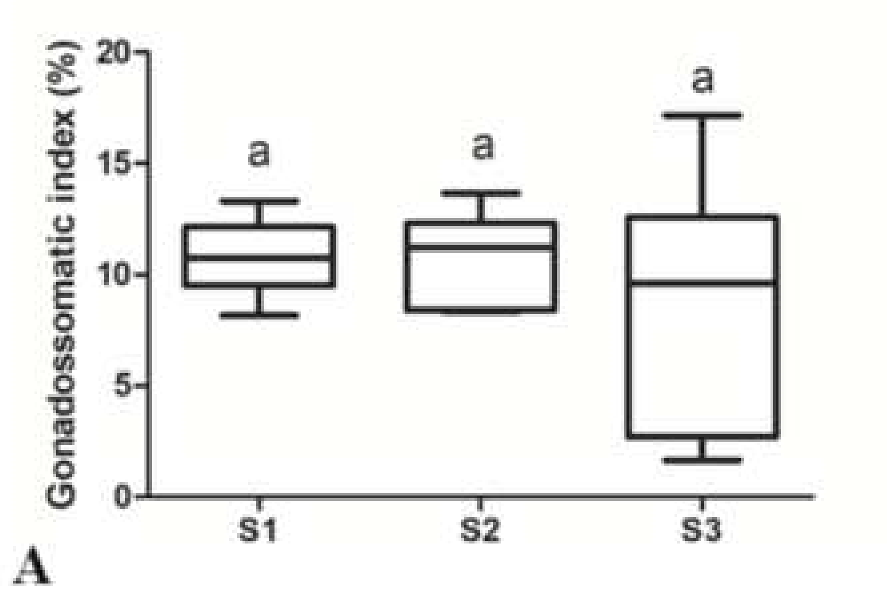

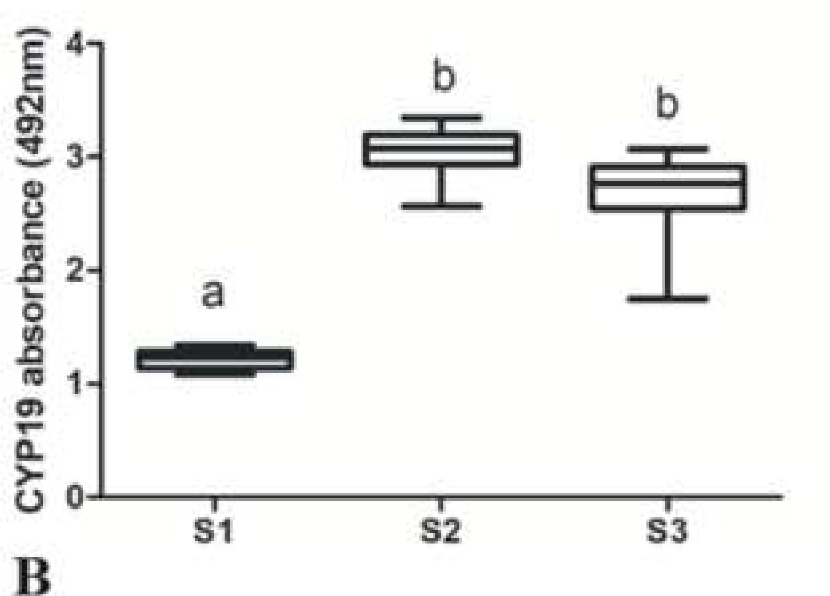

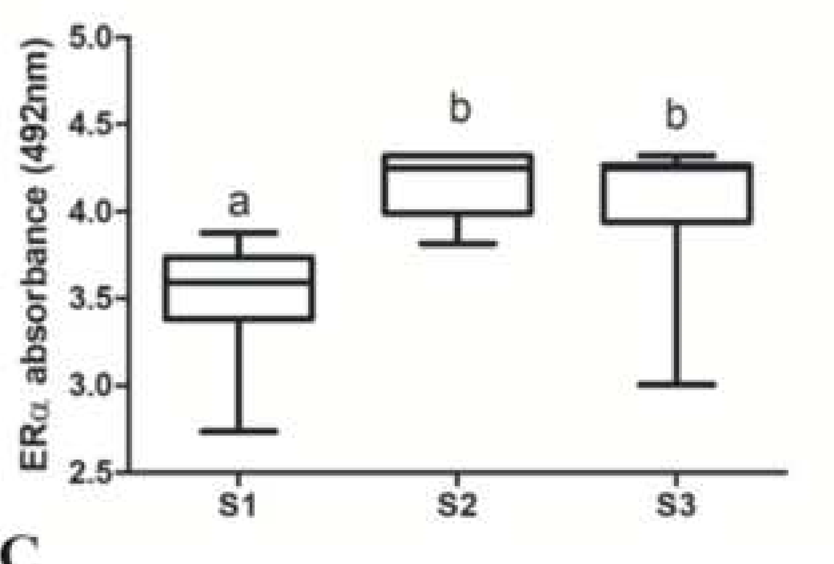

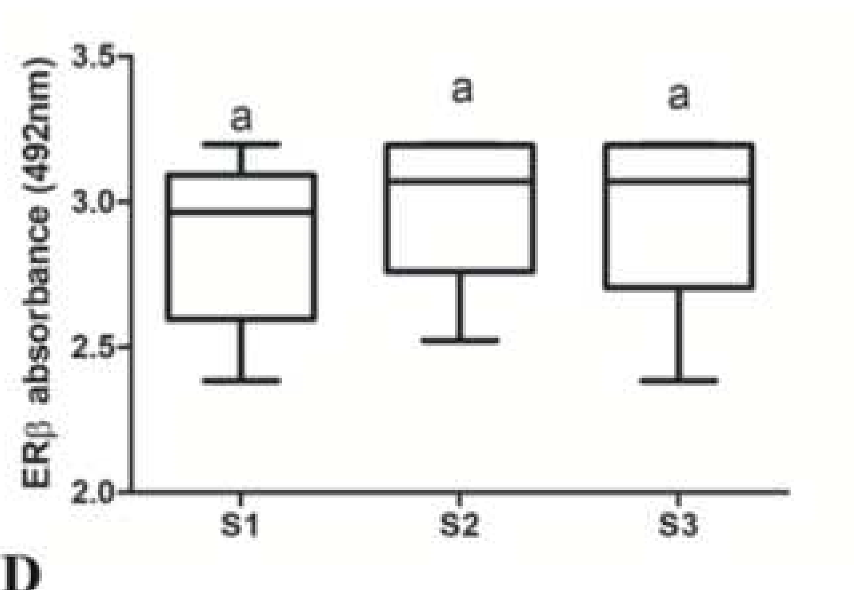

Figure 6

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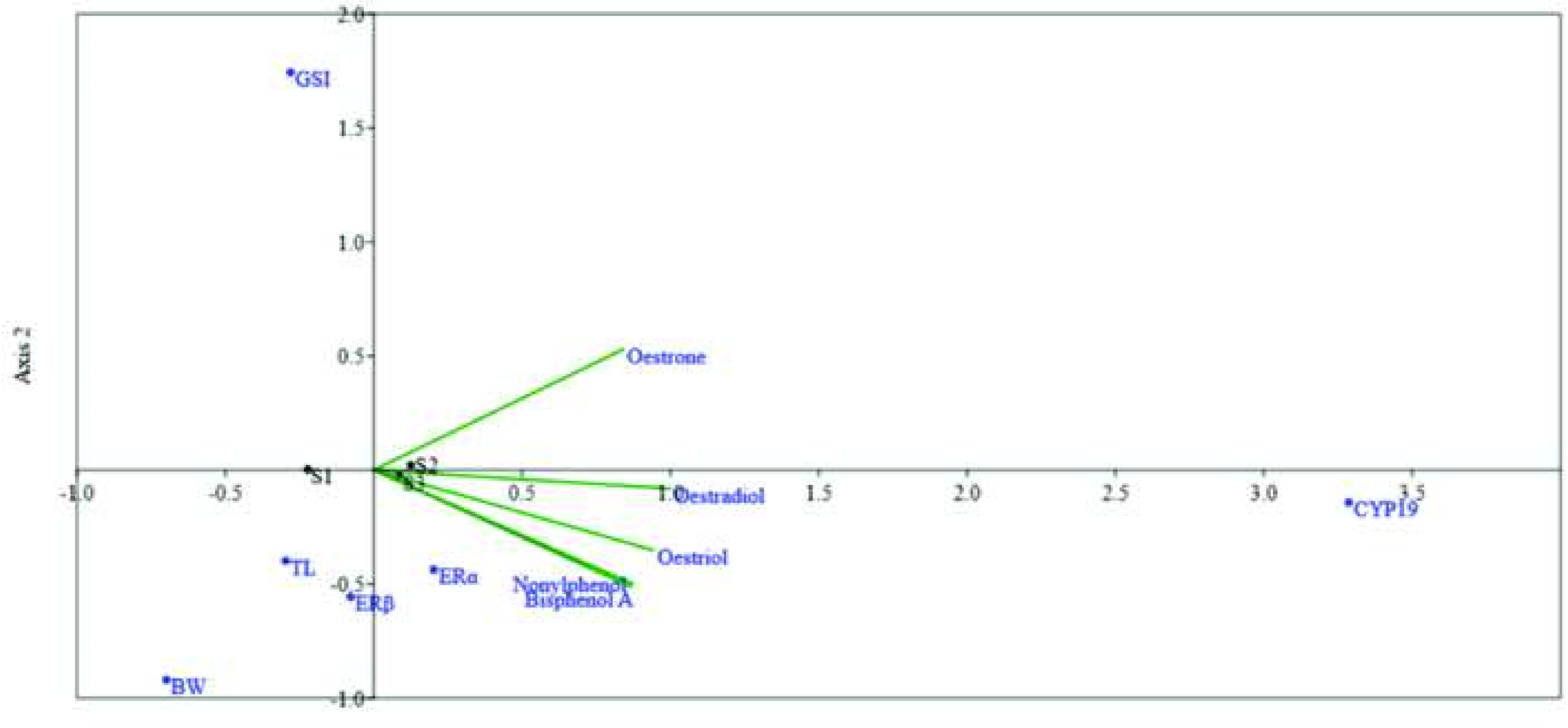

Table

Table 1. Characteristics and pollution sources of the sampling sites.

Site location	Characteristics and impacts
S1 (20°08′50″S; 43°47′43″O)	Reference site with little anthropogenic interference, localized 10 km from urban areas.
S2 (20°06′00″S; 43°47′33″O)	Site exposed to sewage discharges from households and breeding of domestic animals, without any treatment.
S3 (20°05′17″S; 43°47′12″O)	Site located within urban areas, receiving sewage untreated households and homes.

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5. Discussão geral

A bacia do rio das Velhas apresenta uma alta diversidade de espécies de peixes com aproximadamente 130 descritas (Alves and Leal, 2010). Entretanto essa bacia está inserida dentro de uma grande área metropolitana no estado de Minas Gerais recebendo grande quantidade de esgoto doméstico e industrial (Alves and Pompeu, 2005; Moreira et al., 2011; Veado et al., 2000). Projetos de revitalização, como o Projeto Manuelzão, foram criados ao longo dos últimos anos com o propósito de diminuir a carga de poluentes e promover a revitalização dos corpos d'água. No entanto, ainda não existem políticas públicas no Brasil que determinam limites de contaminação para desreguladores endócrinos e fármacos no meio ambiente, o que dificulta a obtenção de resultados favoráveis para a despoluição e revitalização dos rios brasileiros. A ausência/ineficiência de tratamento do esgoto doméstico e industrial, associado à falta de informações dos efeitos dessas substâncias sobre a biota aquática pelos órgãos fiscalizadores governamentais (IBAMA, IEF, SUPRAM) agrava o problema da contaminação aquática no Brasil. Além disso, estudos recentes demonstram que variações da temperatura e pH aumentam a toxicidade de alguns químicos, assim como o levonogestrel (Cardoso et al., 2017). Em um contexto de mudanças climáticas e até mesmo variação sazonal desses parâmetros físico-químicos é importante que essas variações na toxicidade sejam levadas em considerações pelos órgãos fiscalizadores (Almeida et al., 2014). Outro desafio que essa área de estudo promove é a interação entre vários químicos (Silva et al., 2012). O maior número de estudos nessa área analisam os efeitos de EDC's isoladamente, no entanto esses químicos agem em sinergia no ambiente natural, podendo causar diferentes alterações fisiológicas e reprodutivas. Alguns estudos mais recentes abordam a atuação de vários químicos concomitantemente, mas ainda o número de estudos é considerado baixo (Luzio et al., 2015; Silva et al., 2012; Yin et al., 2017), especialmente na América do Sul.

A maioria dos peixes utilizados como modelo em estudos de ecotoxicologia na América do Sul são espécies do gênero *Astyanax* (Paulino et al., 2014; Prado et al., 2014, 2011; Tolussi et al., 2018). As espécies desse gênero apresentam alta abundância de indivíduos, habitam diferentes tipos de ambientes (lagos, lagoas, córregos e rios) e são encontradas em ambientes com diferentes graus de contaminação (Lima et al., 2003). No entanto, a América do Sul apresenta a maior diversidade de peixes do mundo,

com mais de 4.500 espécies descritas de água doce (Almeida et al., 2014). Dessa forma, é importante analisar os efeitos desses contaminantes sobre outras espécies de peixes, uma vez que a resposta a esses contaminantes pode ser diferente dependendo das estratégias utilizadas ao longo do ciclo de vida e das especializações biológicas que essas especies apresentam.

A liberação de contaminantes em ambientes aquáticos é um problema observado em todas as regiões do mundo, podendo causar efeitos adversos para a saúde humana e para as populações de animais e plantas (Adeel et al., 2016; Vázquez et al., 2009). Dentre os contaminantes que afetam os processos fisiológicos, os desreguladores endócrinos (EDC's) são compostos que interferem nos processos de síntese, transporte, ação ou ligação dos hormônios (Denslow and Sepúlveda, 2007). A maioria dos estudos que avaliam os efeitos de EDC's sobre as populações de peixes em ambiente natural, em particular na função reprodutiva, está concentrada em países da Europa (Bjerregaard et al., 2006; Hinck et al., 2007; Jobling et al., 2002; Silva et al., 2012; Tyler and Jobling, 2008), América do Norte (Bahamonde et al., 2015, 2014; Brown et al., 2011; Schultz et al., 2013; Tetreault et al., 2012, 2011) e China (Wen et al., 2013; Zheng et al., 2015). Poucos estudos dessa natureza foram realizados em ecossistemas tropicais, como Brasil (Prado et al., 2014, 2011; Tolussi et al., 2018) e Nigéria (Adeogun et al., 2016; Ibor et al., 2016).

O presente estudo foi realizado em duas etapas. Inicialmente, quantificamos os principais desreguladores endócrinos estrogênicos e avaliamos os efeitos dessa mistura sobre a reprodução da espécie *Astyanax rivularis*, utilizando biomarcadores populacionais, individuais, teciduais e moleculares. Na segunda etapa, realizamos um estudo semi-quantitativo e imunohistoquímico da expressão estágio-específica de CYP19, ERα e ERβ nos testículos de *A. rivularis* e comparamos os níveis testiculares dessas proteínas no pico da maturação gonadal em peixes habitando ambientes com diferentes níveis de contaminação estrogênica em córregos na região do Alto Rio das Velhas.

Na primeira etapa do presente estudo, as análises cromatográficas de EDCs estrogênicos demonstraram uma alta contaminação nos sites de coleta S2 e S3 que recebem esgoto doméstico de cidades e distritos. Por outro lado, o site S1 apresentou baixas concentrações desses compostos nas análises, dessa forma se tornando o site referência do estudo. Em países como EUA e Europa onde ocorre tratamento terciário

dos efluentes domésticos e industriais os níveis de desreguladores endócrinos estrogênicos em rios e córregos são baixos (0.2 – 280 ng/L) (Schultz et al., 2013; Silva et al., 2012). Entretanto, em países subdesenvolvidos ou em desenvolvimento, onde o tratamento do esgoto é apenas primário ou não ocorre tratamento, os níveis dessas substâncias nas águas fluviais são elevados (>3500 ng/L), sendo uma grande ameaça para a biota aquática e para população humana (Adeogun et al., 2016; Ibor et al., 2016; Lopes et al., 2010; Zheng et al., 2015).

No presente trabalho, análise por PCA mostrou que a população de fêmeas do site S1 apresentou melhores condições de saúde e reprodutivas, como valores crescimento corporal, maior proporção de folículos vitelogênicos e menor de folículos perinucleolares. As fêmeas dos sites impactados tiveram deficiência no crescimento corporal, grande proporção de folículos perinucleolares, ovócitos deficientes em vitelo e ovócitos supermaturados, altos valores de LSI e menores níveis de Vtg (Figura 13). Em geral, desreguladores estrogênicos promovem uma deficiência no crescimento de peixes e um aumento no peso do fígado (Prado et al., 2011; Silva et al., 2012). O aumento nos valores de LSI pode estar associado com aumento na síntese de proteínas induzidas e acúmulo de lipídeos que os EDC's promovem (Fishelson, 2006). De forma geral, fêmeas contaminadas por EDC's estrogênicos apresentam menores valores de IGS (Bahamonde et al., 2015; Silva et al., 2012), entretanto no site S3 foi encontrada uma grande proporção de ovócitos supermaturados, o que pode ter ocasionado aumento no valor de IGS e maior fecundidade. Cabe destacar que os ovócitos supermaturados são ovócitos envelhecidos que não foram ovulados, provavelmente devido ao atraso na troca esteroidogênica que leva a maturação final ovocitária (Prado et al., 2014). Esse resultado demonstra que os EDC's agem de diferentes formas dependendo da composição e concentração das substâncias tóxicas presente na mistura de contaminantes (Prado et al., 2014).

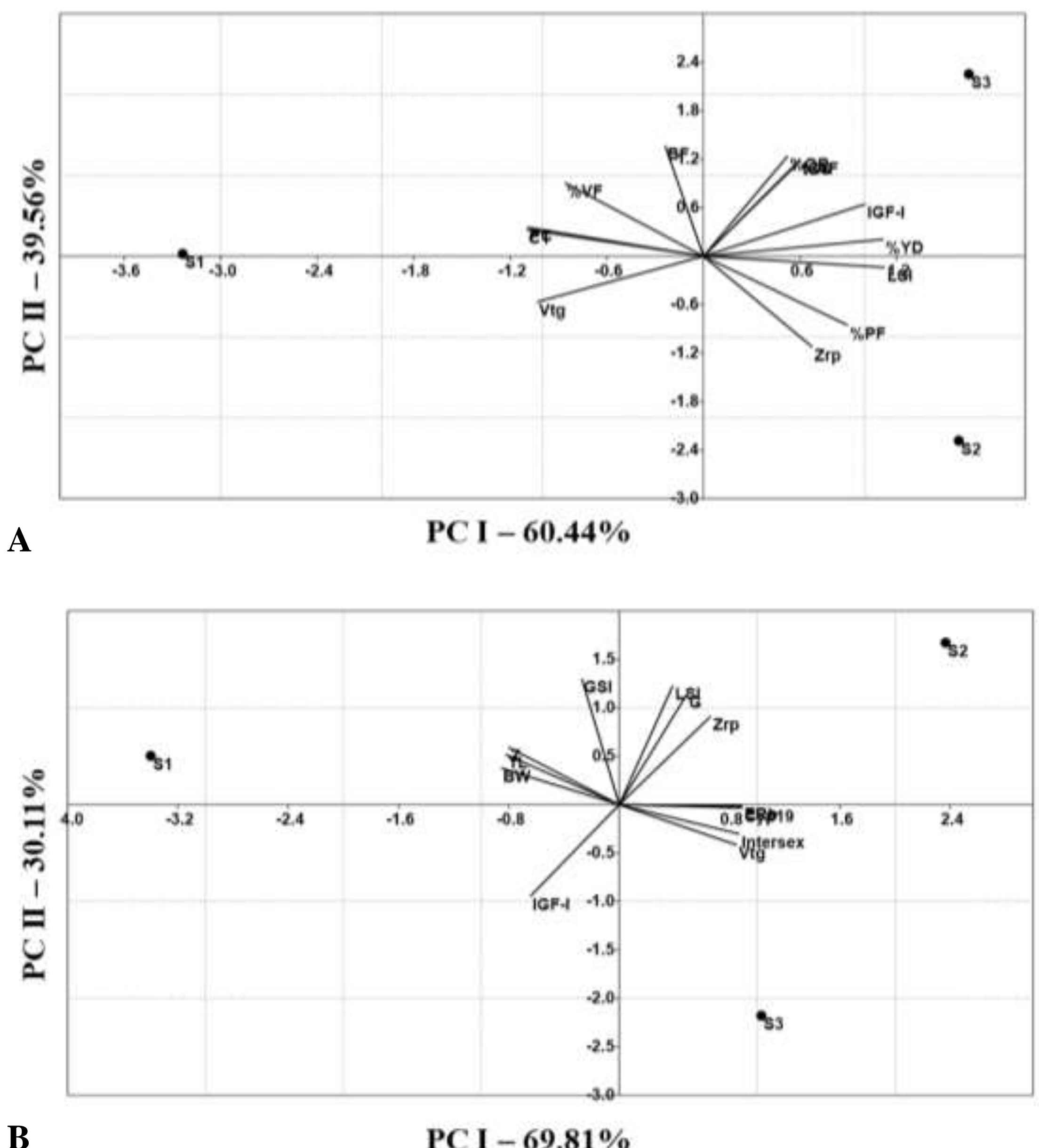

Figura 1 – Análise de PCA mostrando diferenças nas populações de fêmeas (A) e de machos (B) de *A. rivularis* nos diferentes sites do Alto Rio das Velhas.

Em relação aos machos, a população do site S1 seguiu mesma tendência das fêmeas com maior crescimento corporal e também uma maior proporção de espermatozoides. Nos sites S2 e S3 os indivíduos são menores e menos pesados. Em ambos os sites foi observado processo de feminilização, como aumento significativo da proporção de fêmeas em relação aos machos, intersexo e também maiores níveis de Vtg hepática (**Erro! Fonte de referência não encontrada.**). No presente estudo, os

indivíduos intersexo apresentaram apenas folículos perinucleolares e a proporção sexual foi de 6.5 fêmeas para cada macho em S2 e 3.2 fêmeas para cada macho em S3. Em rios do Canadá foram observados maiores níveis de desregulação estrogênica na espécie *Etheostoma caeruleum*, com proporção sexual de até 10 fêmeas para cada macho e a presença de peixes intersexo, tendo até 50% da gônada preenchida por ovócitos, incluindo a presença de folículos vitelogênicos-(Bahamonde et al., 2015).

A maioria dos estudos de campo demonstra aumento de Vtg hepática em machos que habitam regiões contaminadas por esgoto doméstico (Bahamonde et al., 2014; Prado et al., 2014; Schultz et al., 2013), indicando Vtg como um excelente biomarcador de desregulação endócrina por estrógenos ambientais como também detectado no presente estudo para o Alto Rio das Velhas. Por outro lado, expressão de Zrp precede a da Vtg, sugerindo que Zrp tem uma maior sensibilidade à indução estrogênica (Arukue and Roe, 2008) sendo capaz de detecção precoce da contaminação estrogênica (Prado et al., 2011), entretanto não foi detectada diferença significativa em machos entre os sites do presente estudo. Além disso, valores significativamente reduzidos de IGF-I hepático foram detectados para machos de S2, onde os peixes apresentaram menor tamanho corporal. Esses resultados indicam Vtg e IGF-I como apropriados biomarcadores moleculares de desregulação endócrina estrogênica para o Rio das Velhas.

Na segunda etapa do projeto, foi realizada uma análise estágio-específica dos níveis testiculares de ERα, ERβ e CYP19 pela primeira vez em uma espécie de peixe Neotropical. Além disso, foi avaliado os níveis dessas proteínas em machos submetidos a contaminação por esgoto doméstico. Os resultados demonstraram imunomarcação de CYP19 em células intersticiais como granulócitos acidófilos e células de Leydig e também algumas células germinativas como espermatogônias, espermátides e espermatozoides. De fato, os estrógenos participam na homeostase do ácido retinóico dentro dos testículos, sendo essa molécula importante na proliferação e diferenciação de espermatogônias (Zhou et al., 2008) e também estudos com mamíferos sugerem uma possível participação de estrógenos na geração de energia para motilidade espermática (Carreau and Hess, 2010). Além da marcação da linhagem germinativa, estudos com a espécie *Sparus aurata*, em estágio gonadal pós-espermiado, demonstram que alguns leucócitos, como granulócitos acidófilos presentes nos testículos são capazes de expressar CYP19, demonstrando que essas células participam da retomada da

espermatogênese (Chaves-Pozo et al., 2007). Entretanto, essas células não expressão nenhum ER (Liarte et al., 2007).

Estudos de imunolocalização de receptores de estrógeno (ERs) em peixes são escassos na literatura. Os resultados do presente estudo demonstraram que o ERβ apresenta maior especificidade do que o ERα. Estudos com a espécie *Dicentrachus labrax* apresentaram resultados semelhantes aos do presente estudo, com expressão de ERα em todas as células germinativas e ERβ apenas em espermatogônias e espermatócitos (Viñas and Piferrer, 2008). Os ER's apresentam padrões de expressão semelhantes ao longo da maturação gonadal demonstrando que eles não possuem funções antagônicas, assim como observado na truta arco-íris (Delalande et al., 2015). Embora tenham similar dominios de ligação ao ligante e ao DNA, ERα e ERβ possuem algumas propriedades únicas em termos de seletividade do ligante e regulação do gene alvo (Shanle and Xu, 2011).

Os machos dos sites S2 e S3 apresentaram maiores níveis de ERα e CYP19 em relação aos machos do site referência S1. Estudo com *Gambusia affinis* em rios contaminados por esgoto doméstico na China demonstraram aumento significante de ERα hepático em machos e fêmeas (Wen et al., 2013). Da mesma forma, em rios contaminados por bifenilas policloradas (PCB's) mostraram aumento de CYP19 hepático em duas espécies de tilápias, sendo a expressão de CYP19 em machos maior que a expressão em fêmeas (Ibor et al., 2016). Entretanto, estudo realizado nos testículos de peixes intersexo de *Etheostoma caeruleum* em rios do Canadá não apresentou aumento nos níveis de receptores nucleares de estrógeno (Bahamonde et al., 2014).

Os ERs têm grandes sítios ('pockets') de ligação e são considerados receptores nucleares versáteis e relativamente promíscuos devido à afinidade com diversos ligantes exógenos (Ng et al., 2014), tornando as células muito vulneráveis à ação de diversos contaminantes. Os EDCs podem atuar na sinalização genômica e não genômica dos ERs através de interações diretas com ERs, incluindo produtos químicos farmacêuticos, bisfenóis, fitoestrógenos e pesticidas organoclorados. Frequentemente, os EDCs atuam através de múltiplos mecanismos, incluindo a ação indireta através de fatores de transcrição como o receptor de aril hidrocarboneto (AhR), ou através da modulação de enzimas metabólicas que são críticas para síntese e metabolismo normal do estrógeno (Marino et al., 2006; Shanle and Xu, 2011). Conhecidos EDCs como bisfenol A (BPA)

e dietilestilbestrol (DES) induzem uma sinalização estrogênica rápida, através da atuação na via de sinalização ER não genômica (Nadal et al., 2000).

O uso de diferentes tipos de biomarcadores em vários níveis de organização biológica permitiu não apenas a detecção das alterações no processo reprodutivo de *A. rivularis*, mas também confirmou a presença de EDCs estrogênicos no Alto Rio das Velhas, em uma área importante de preservação ambiental. A contaminação de corpos d'água é um problema observado em praticamente em todos os países do mundo, sendo mais preocupante na América do Sul onde os efluentes domésticos e industriais não são tratados da forma adequada para remoção desses contaminantes. É importante destacar a necessidade de maior investimento público e privado no Brasil e estudos científicos na área da ecotoxicologia ambiental para fomentar as politicas públicas de regulamentação dos níveis de EDCs nos corpos d'água, visando à conservação dos ecossistemas aquáticos de água doce e a ictiofauna neotropical.

6. Conclusões

- Os elevados níveis de desreguladores endócrinos estrogênicos presentes nos sites S2 e S3, decorrente do despejo de esgoto doméstico, está afetando a reprodução da espécie *A. rivularis*, no alto rio das Velhas;
- Os machos e fêmeas de *A. rivularis* que habitam ambientes contaminados por esgoto doméstico tiveram seus níveis de Vtg, Zrp, ERα, CYP19 e IGF-I alterados;
- No ponto referência, S1, com pouca interferência antrópica os parâmetros reprodutivos e de saúde foram adequados para a reprodução da espécie;
- A imunolocalização de CYP19, ERα e ERβ em testículos é estágio-específica em *A. rivularis* e as três proteínas apresentam padrões semelhantes de expressão ao longo do desenvolvimento gonadal.

7. Referências bibliográficas

Adeel, M., Song, X., Wang, Y., Francis, D., Yang, Y., 2016. Environmental impact of estrogens on human, animal and plant life: A critical review. Environ. Int. 99, 107–119. doi:10.1016/j.envint.2016.12.010

Adeogun, A.O., Onibonoje, K., Ibor, O.R., Omiwole, R.A., Chukwuka, A.V., Ugwumba, A.O., Ugwumba, A.A.A., Arukwe, A., 2016. Endocrine-disruptor molecular responses, occurrence of intersex and gonado-histopathological changes in tilapia species from a tropical freshwater dam (Awba Dam) in Ibadan, Nigeria. Aquat. Toxicol. 174, 10–21.

Almeida, E.A. De, Rodrigues, A.C.F., de Oliveira Ribeiro, C.A., 2014. Introduction—Pollution and Fish Health in Tropical Ecosystems: A Brief Summary on Current Challenges and Perspectives, in: Almeida, E.A. De, de Oliveira Ribeiro, C.A. (Eds.), Pollution and Fish Health in Tropical Ecosystems. Taylor & Francis, London, pp. 1–14.

Alves, C.B.M., Leal, C.G., 2010. Aspectos da conservacão da fauna de peixes da bacia do rio São Francisco em Minas Gerais. MG Biota 2, 26–44.

Alves, C.B.M., Pompeu, P.S., 2005. Historical Changes in the Rio das Velhas Fish Fauna—Brazil. Am. Fish. Soc. Symp. 45, 587–602.

Ankley, G.T., Kahl, M.D., Jensen, K.M., Hornung, M.W., Korte, J.J., Makynen, E.A., Leino, R.L., 2002. Evaluation of the aromatase inhibitor fadrozole in a short-term reproduction assay with the fathead minnow (*Pimephales promelas*). Toxicol. Sci. 67, 121–130.

Aranda, A., Pascual, A., 2001. Nuclear hormone receptors and gene expression. Physiol. Rev. 81, 1269–304. doi:0031-9333/01

Atkinson, S.K., 2008. The persistence of stereoidal estrogen in the aquatic envionment. Universidade de Ottawa.

Babin, P.J., Carnevali, O., Lubzens, E., Schneider, W.J., 2007. Molecular aspects of oocyte vitellogenesis in fish, in: Babin, P.J., Cerdà, J., Lubzens, E. (Eds.), The Fish Oocyte: From Basic Studies to Biotechnological Applications. Springer, Dordrecht, pp. 39–76.

Bahamonde, P.A., Fuzzen, M.L., Bennett, C.J., Tetreault, G.R., McMaster, M.E., Servos, M.R., Martyniuk, C.J., Munkittrick, K.R., 2015. Whole organism responses and intersex severity in rainbow darter (*Etheostoma caeruleum*) following exposures to municipal wastewater in the Grand River basin, ON, Canada. Part A. Aquat. Toxicol. 159, 290–301.

Bahamonde, P.A., Munkittrick, K.R., Martyniuk, C.J., 2013. Intersex in teleost fish: Are we distinguishing endocrine disruption from natural phenomena? Gen. Comp. Endocrinol. 192, 25–35.

Bahamonde, P.A., Tetreault, G.R., McMaster, M.E., Servos, M.R., Martyniuk, C.J., Munkittrick, K.R., 2014. Molecular signatures in rainbow darter (*Etheostoma caeruleum*) inhabiting an urbanized river reach receiving wastewater effluents. Aquat. Toxicol. 148, 211–220. doi:10.1016/j.aquatox.2014.01.010

Bjerregaard, L.B., Korsgaard, B., Bjerregaard, P., 2006. Intersex in wild roach (*Rutilus rutilus*) from Danish sewage effluent-receiving streams. Ecotoxicol. Environ. Saf. 64, 321–328.

Bolong, N., Ismail, A.F., Salim, M.R., Matsuura, T., 2009. A review of the effects of emerging contaminants in wastewater and options for their removal. Desalination 238, 229–246. doi:10.1016/j.desal.2008.03.020

Brown, C.J.M., Knight, B.W., McMaster, M.E., Munkittrick, K.R., Oakes, K.D., Tetreault, G.R., Servos, M.R., 2011. The effects of tertiary treated municipal wastewater on fish communities of a small river tributary in Southern Ontario, Canada. Environ. Pollut. 159, 1923–1931. doi:10.1016/j.envpol.2011.03.014

Burgos-Aceves, M.A., Cohen, A., Smith, Y., Faggio, C., 2016. Estrogen regulation of gene expression in the teleost fish immune system. Fish Shellfish Immunol. 58, 42–49. doi:10.1016/j.fsi.2016.09.006

Callard, G. V., Tchoudakova, A. V., Kishida, M., Wood, E., 2001. Differential tissue distribution, developmental programming, estrogen regulation and promoter characteristics of cyp19 genes in teleost fish. J. Steroid Biochem. Mol. Biol. 79, 305–314. doi:10.1016/S0960-0760(01)00147-9

Campbell, B., 2003. Endocrine Changes During Onset of Puberty in Male Spring Chinook Salmon, Oncorhynchus tshawytscha. Biol. Reprod. 69, 2109–2117.

Cardoso, P.G., Rodrigues, D., Madureira, T. V., Oliveira, N., Rocha, M.J., Rocha, E., 2017. Warming modulates the effects of the endocrine disruptor progestin levonorgestrel on the zebrafish fitness, ovary maturation kinetics and reproduction success. Environ. Pollut. 229, 300–311. doi:10.1016/j.envpol.2017.05.090

Carnevali, O., Carletta, R., Cambi, A., Vita, A., Bromage, N., 1999. Yolk formation and degradation during oocyte maturation in seabream *Sparus aurata*: involvement of two lysosomal proteinases. Biol. Reprod. 60, 140–146.

Carreau, S., Hess, R.A., 2010. Oestrogens and spermatogenesis. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 365, 1517–35. doi:10.1098/rstb.2009.0235

Chaves-Pozo, E., Liarte, S., Vargas-Chacoff, L., García-López, A., Mulero, V., Meseguer, J., Mancera, J.M., García-Ayala, A., 2007. 17Beta-Estradiol Triggers Postspawning in Spermatogenically Active Gilthead Seabream (*Sparus aurata* L.) Males1. Biol. Reprod. 76, 142–148. doi:10.1095/biolreprod.106.056036

de Voogt, P., van Hattum, B., 2003. Critical factors in exposure modeling of endocrine active substances. Pure Appl. Chem. 75, 1933–1948.

Delalande, C., Goupil, A.S., Lareyre, J.J., Le Gac, F., 2015. Differential expression patterns of three aromatase genes and of four estrogen receptors genes in the testes of trout (*Oncorhynchus mykiss*). Mol. Reprod. Dev. 82, 694–708. doi:10.1002/mrd.22509

Denslow, N., Sepúlveda, M., 2007. Ecotoxicological effects of endocrine disrupting compounds on fish reproduction, in: Babin, P.J., Cerdà, J., Lubzens, E. (Eds.), The Fish Oocyte: From Basic Studies to Biotechnological Applications. Springer, Dordrecht, pp. 255–322.

Filardo, E.J., 2002. Epidermal growth factor receptor (EGFR) transactivation by

estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer. J. Steroid Biochem. Mol. Biol. 80, 231–238. doi:10.1016/S0960-0760(01)00190-X

Fishelson, L., 2006. Cytomorphological alterations of the thymus, spleen, head-kidney, and liver in cardinal fish (Apogonidae, Teleostei) as bioindicators of stress. J. Morphol. 267, 57–69.

Folkertsma, S., Van Noort, P., Van Durme, J., Joosten, H.J., Bettler, E., Fleuren, W., Oliveira, L., Horn, F., De Vlieg, J., Vriend, G., 2004. A family-based approach reveals the function of residues in the nuclear receptor ligand-binding domain. J. Mol. Biol. doi:10.1016/j.jmb.2004.05.075

Fuzzen, M.L.M., Bennett, C.J., Tetreault, G.R., McMaster, M.E., Servos, M.R., 2015. Severe intersex is predictive of poor fertilization success in populations of rainbow darter (*Etheostoma caeruleum*). Aquat. Toxicol. 160, 106–116.

Grabicova, K., Lindberg, R.H., Östman, M., Grabic, R., Randak, T., Joakim Larsson, D.G., Fick, J., 2014. Tissue-specific bioconcentration of antidepressants in fish exposed to effluent from a municipal sewage treatment plant. Sci. Total Environ. 488–489, 46–50.

Gustafsson, J.Å., 2003. What pharmacologists can learn from recent advances in estrogen signalling. Trends Pharmacol. Sci. 24, 479–485. doi:10.1016/S0165-6147(03)00229-3

Harris, J., Bird, D.J., 2000. Modulation of the fish immune system by hormones. Vet. Immunol. Immunopathol. 77, 163–176. doi:10.1016/S0165-2427(00)00235-X

Hess, R.A., 2003. Estrogen in the adult male reproductive tract: a review. Reprod. Biol. Endocrinol. 1, 52. doi:10.1186/1477-7827-1-52

Hinck, J.E., Blazer, V.S., Denslow, N.D., Echols, K.R., Gross, T.S., May, T.W., Anderson, P.J., Coyle, J.J., Tillitt, D.E., 2007. Chemical contaminants, health indicators, and reproductive biomarker responses in fish from the Colorado River and its tributaries. Sci. Total Environ. 378, 376–402. doi:10.1016/j.scitotenv.2007.02.032

Huhtaniemi, I.T., Themmen, A.P.N., 2005. Mutations in human gonadotropin and gonadotropin-receptor genes. Endocrine 26, 207–217.

Hull, K.L., Harvey, S., 2014. Growth Hormone and Reproduction: A Review of Endocrine and Autocrine/Paracrine Interactions. Int. J. Endocrinol. 2014, 1–24.

IBGE, 2014. Sinopse do Censo e Resultados Preliminares do Universo [WWW Document]. URL http://www.ibge.gov.br/home/ (accessed 6.21.16).

Ibor, O.R., Adeogun, A.O., Fagbohun, O.A., Arukwe, A., 2016. Gonado-histopathological changes, intersex and endocrine disruptor responses in relation to contaminant burden in Tilapia species from Ogun River, Nigeria. Chemosphere 164, 248–262.

Jobling, S., Coey, S., Whitmore, J.G., Kime, D.E., Van Look, K.J.W., McAllister, B.G., Beresford, N., Henshaw, A.C., Brighty, G., Tyler, C.R., Sumpter, J.P., 2002. Wild Intersex Roach (*Rutilus rutilus*) Have Reduced Fertility. Biol. Reprod. 67, 515–524.

Kagawa, H., Gen, K., Okuzawa, K., Tanaka, H., 2003. Effects of luteinizing hormone and follicle-stimulating hormone and insulin-like growth factor-I on aromatase activity and P450 aromatase gene expression in the ovarian follicles of red seabream, *Pagrus major*. Biol. Reprod. 68, 1562–1568.

Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D., Chambon, P., 1995. Activation of the Estrogen Receptor Through Phosphorylation by Mitogen-Activated Protein Kinase. Science, 270, 1491–1494. doi:10.1126/science.270.5241.1491

Kerdivel, G., Habauzit, D., Pakdel, F., 2013. Assessment and molecular actions of endocrine-disrupting chemicals that interfere with estrogen receptor pathways. Int. J. Endocrinol. doi:10.1155/2013/501851

Kloas, W., Schrag, B., Ehnes, C., Segner, H., 2000. Binding of xenobiotics to hepatic estrogen receptor and plasma sex steroid binding protein in the teleost fish, the common carp (*Cyprinus carpio*). Gen. Comp. Endocrinol. 119, 287–99. doi:10.1006/gcen.2000.7521

Lange, A., Katsu, Y., Ichikawa, R., Paull, G.C., Chidgey, L.L., Coe, T.S., Iguchi, T., Tyler, C.R., 2008. Altered sexual development in roach (*Rutilus rutilus*) exposed to environmental concentrations of the pharmaceutical 17α-Ethinylestradiol and associated expression dynamics of aromatases and estrogen receptors. Toxicol. Sci. 106, 113–123.

Liarte, S., Chaves-Pozo, E., García-Alcazar, A., Mulero, V., Meseguer, J., García-Ayala, A., 2007. Testicular involution prior to sex change in gilthead seabream is characterized by a decrease in DMRT1 gene expression and by massive leukocyte infiltration. Reprod. Biol. Endocrinol. 5, 20. doi:10.1186/1477-7827-5-20

Lima, F.C.T., Malabarba, L.R., Buckup, P.A., Pezzi da Silva, J.F., Vari, R.P., Harold, A., Benine, R., Oyakawa, O.T., Pavanelli, C.S., Menezes, N.A., Lucena, C.A.S., Malabarba, M.C.S.L., Lucena, Z.M.S., Reis, R.E., Langeani, F., Moreira, C., 2003. Genera Incertae Sedis in Characidae, in: Reis, R.E., Kullander, S.O., Ferraris-Jr, C.J. (Eds.), Check List of the Freshwater Fishes of South and Central America. EDIPUCRS, Porto Alegre, pp. 106–168.

Lintelmann, J., Katayama, A., Kurihara, N., Shore, L., Wenzel, A., 2003. Endocrine disruptors in the environment. Pure Appl. Chem. 75, 631–681.

Lopes, L.G., Marchi, M.R.R., Souza, J.B.G., Moura, J.A., Lorenzon, C.S., Cruz, C., Amaral, L.A., 2010. Estrogênios em águas naturais e tratadas da região de jaboticabal - São Paulo. Quim. Nova 33, 639–643. doi:10.1590/S0100-40422010000300029

Lubzens, E., Young, G., Bobe, J., Cerdà, J., 2010. Oogenesis in teleosts: How fish eggs are formed. Gen. Comp. Endocrinol. 165, 367–389.

Luzio, A., Monteiro, S.M., Garcia-Santos, S., Rocha, E., Fontaínhas-Fernandes, A.A., Coimbra, A.M., 2015. Zebrafish sex differentiation and gonad development after exposure to 17α-ethinylestradiol, fadrozole and their binary mixture: A stereological study. Aquat. Toxicol. 166, 83–95.

Luzio, A., Monteiro, S.M., Rocha, E., Fontaínhas-Fernandes, A.A., Coimbra, A.M.,

2016. Development and recovery of histopathological alterations in the gonads of zebrafish (*Danio rerio*) after single and combined exposure to endocrine disruptors (17α-ethinylestradiol and fadrozole). Aquat. Toxicol. 175, 90–105.

Madureira, T.V., Rocha, M.J., Cruzeiro, C., Rodrigues, I., Monteiro, R.A.F., Rocha, E., 2012. The toxicity potential of pharmaceuticals found in the Douro River estuary (Portugal): Evaluation of impacts on fish liver, by histopathology, stereology, vitellogenin and CYP1A immunohistochemistry, after sub-acute exposures of the zebrafish model. Environ. Toxicol. Pharmacol. 34, 34–45.

Marino, M., Galluzzo, P., Ascenzi, P., 2006. Estrogen signaling multiple pathways to impact gene transcription. Curr Genomics 7, 497–508. doi:10.2174/138920206779315737

Modig, C., Westerlund, L., Olsson, P.E., 2007. Oocyte zona pellucida proteins, in: Babin, P.J., Cerdà, J., Lubzens, E. (Eds.), The Fish Oocyte: From Basic Studies to Biotechnological Applications. Springer, Dordrecht, pp. 113–139.

Moreira, M., Aquino, S., Coutrim, M., Silva, J., Afonso, R., 2011. Determination of endocrine disrupting compounds in waters from Rio das Velhas, Brazil, by liquid chromatography/high resolution mass spectrometry (ESI-LC-IT-TOF/MS). Environ. Technol. 32, 1409–1417. doi:10.1080/09593330.2010.537829

Myers, N., Mittermeier, R.A., Mittermeier, C.G., da Fonseca, G.A., Kent, J., 2000. Biodiversity hotspots for conservation priorities. Nature 403, 853–858. doi:10.1038/35002501

Nadal, A., Ropero, A.B., Laribi, O., Maillet, M., Fuentes, E., Soria, B., 2000. Nongenomic actions of estrogens and xenoestrogens by binding at a plasma membrane receptor unrelated to estrogen receptor alpha and estrogen receptor beta. Proc. Natl. Acad. Sci. U. S. A. 97, 11603–8. doi:10.1073/pnas.97.21.11603

Nakamura, I., Kusakabe, M., Young, G., 2003. Regulation of steriodogenic enzyme mRNAs in rainbow trout (*Oncorhynchus mykiss*) ovarian follicles in vitro. Fish Physiol. Biochem. 28, 355–356.

Nelson, E.R., Habibi, H.R., 2013. Estrogen receptor function and regulation in fish and other vertebrates. Gen. Comp. Endocrinol. 192, 15–24. doi:10.1016/j.ygcen.2013.03.032

Ng, H.W., Perkins, R., Tong, W., Hong, H., 2014. Versatility or promiscuity: The estrogen receptors, control of ligand selectivity and an update on subtype selective ligands. Int. J. Environ. Res. Public Health. doi:10.3390/ijerph110908709

O'Malley, B.W., Tsai, M.J., 1992. Molecular pathways of steroid receptor action. Biol. Reprod. 46, 163–167. doi:10.1095/biolreprod46.2.163

OECD, 2009. OECD Guidance document for the diagnosis of endocrine-related histopathology of fish gonads, OECD Environment, Health and Safety Publications, Series on Testing and Assessment.

Paulino, M.G., Benze, T.P., Sadauskas-Henrique, H., Sakuragui, M.M., Fernandes, J.B., Fernandes, M.N., 2014. The impact of organochlorines and metals on wild fish living in a tropical hydroelectric reservoir: Bioaccumulation and histopathological biomarkers. Sci. Total Environ. 497–498, 293–306.

doi:10.1016/j.scitotenv.2014.07.122

Pierce, A.L., Shimizu, M., Beckman, B.R., Baker, D.M., Dickhoff, W.W., 2005. Time course of the GH/IGF axis response to fasting and increased ration in chinook salmon (*Oncorhynchus tshawytscha*). Gen. Comp. Endocrinol. 140, 192–202.

Prado, P.S., Pinheiro, A.P.B., Bazzoli, N., Rizzo, E., 2014. Reproductive biomarkers responses induced by xenoestrogens in the characid fish *Astyanax fasciatus* inhabiting a South American reservoir: An integrated field and laboratory approach. Environ. Res. 131, 165–173. doi:10.1016/j.envres.2014.03.002

Prado, P.S., Souza, C.C., Bazzoli, N., Rizzo, E., 2011. Reproductive disruption in lambari *Astyanax fasciatus* from a Southeastern Brazilian reservoir. Ecotoxicol. Environ. Saf. 74, 1879–1887. doi:10.1016/j.ecoenv.2011.07.017

Rasmussen, T.H., Korsgaard, B., 2004. Estrogenic octylphenol affects seminal fluid production and its biochemical composition of eelpout (*Zoarces viviparus*). Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 139, 1–10.

Redding, J.M., Patino, R., 1993. Reproductive physiology, in: Evans, D.H. (Ed.), Physiology of Fishes. CRC Press, Boca Raton, pp. 503–534.

Reinecke, M., 2010. Insulin-like growth factors and fish reproduction. Biol. Reprod. 82, 656–661.

Robertson, K.M., Simpson, E.R., Lacham-Kaplan, O., Jones, M.E., 2001. Characterization of the fertility of male aromatase knockout mice. J. Androl. 22, 825–830.

Santos, M.M., Ruivo, R., Lopes-Marques, M., Torres, T., Santos, C.B. d. los, Castro, F.C., Neuparth, T., 2016. Statins: as undesirable class os aquatic contaminants? Aquat. Toxicol. doi:10.1016/j.aquatox.2016.02.001

Schultz, M.M., Minarik, T.A., Martinovic-Weigelt, D., Curran, E.M., Bartell, S.E., Schoenfuss, H.L., 2013. Environmental estrogens in an urban aquatic ecosystem: II. Biological effects. Environ. Int. 61, 138–149. doi:10.1016/j.envint.2013.08.006

Schulz, R.W., França, L.R., Lareyre, J.J., LeGac, F., Chiarini-Garcia, H., Nobrega, R.H., Miura, T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165, 390–411. doi:10.1016/j.ygcen.2009.02.013

Schulz, U.H., Martins-Junior, H., 2001. Astyanax fasciatus as bioindicator of water pollution of Rio dos Sinos, RS, Brazil. Brazilian J. Biol. 61, 615–22.

Schwabe, J.W., Chapman, L., Rhodes, D., 1995. The oestrogen receptor recognizes an imperfectly palindromic response element through an alternative side-chain conformation. Structure 3, 201–213. doi:10.1016/S0969-2126(01)00150-2

Shanle, E.K., Xu, W., 2011. Endocrine disrupting chemicals targeting estrogen receptor signaling: Identification and mechanisms of action. Chem. Res. Toxicol. doi:10.1021/tx100231n

Shibatta, O.A., Cheida, C.C., 2003. Composição em tamanho dos peixes (Actinopterygii, Teleostei) de ribeirões da bacia do rio Tibagi, Paraná, Brasil. Rev. Bras. Zool. 20, 469–473.

Silva, P., Rocha, M.J., Cruzeiro, C., Malhão, F., Reis, B., Urbatzka, R., Monteiro,

R.A.F., Rocha, E., 2012. Testing the effects of ethinylestradiol and of an environmentally relevant mixture of xenoestrogens as found in the Douro River (Portugal) on the maturation of fish gonads-A stereological study using the zebrafish (*Danio rerio*) as model. Aquat. Toxicol. 124–125, 1–10. doi:10.1016/j.aquatox.2012.07.002

Sohoni, P., Tyler, C.R., Hurd, K., Caunter, J., Hetheridge, M., Williams, T., Woods, C., Evans, M., Toy, R., Gargas, M., Sumpter, J.P., 2001. Reproductive effects of long-term exposure to Bisphenol A in the fathead minnow (*Pimephales promelas*). Environ. Sci. Technol. 35, 2917–2925.

Sumpter, J.P., Jobling, S., 2013. The occurrence, causes, and consequences of estrogens in the aquatic environment. Environ. Toxicol. Chem. 32, 249–251. doi:10.1002/etc.2084

Szego, C.M., Davis, J.S., 1967. Adenosine 3',5'-monophosphate in rat uterus: acute elevation by estrogen. Proc. Natl. Acad. Sci. U. S. A. 58, 1711–8. doi:10.1073/pnas.58.4.1711

Tetreault, G.R., Bennett, C.J., Cheng, C., Servos, M.R., McMaster, M.E., 2012. Reproductive and histopathological effects in wild fish inhabiting an effluent-dominated stream, Wascana Creek, SK, Canada. Aquat. Toxicol. 110–111, 149–161. doi:10.1016/j.aquatox.2012.01.004

Tetreault, G.R., Bennett, C.J., Shires, K., Knight, B., Servos, M.R., McMaster, M.E., 2011. Intersex and reproductive impairment of wild fish exposed to multiple municipal wastewater discharges. Aquat. Toxicol. 104, 278–290. doi:10.1016/j.aquatox.2011.05.008

Thomas, P., 2012. Rapid steroid hormone actions initiated at the cell surface and the receptors that mediate them with an emphasis on recent progress in fish models. Gen. Comp. Endocrinol. 175, 367–383. doi:10.1016/j.ygcen.2011.11.032

Tolussi, C.E., Gomes, A.D.O., Kumar, A., Ribeiro, C.S., Nostro, F.L.L., Bain, P.A., de Souza, G.B., Cuña, R. Da, Honji, R.M., Moreira, R.G., 2018. Environmental pollution affects molecular and biochemical responses during gonadal maturation of *Astyanax fasciatus* (Teleostei: Characiformes: Characidae). Ecotoxicol. Environ. Saf. 147, 926–934. doi:10.1016/j.ecoenv.2017.09.056

Tyler, C.R., Jobling, S., 2008. Roach, Sex, and Gender-Bending Chemicals: The Feminization of Wild Fish in English Rivers. Bioscience 58, 1051–1059.

Vázquez, G.R., Meijide, F.J., Da Cuña, R.H., Lo Nostro, F.L., Piazza, Y.G., Babay, P.A., Trudeau, V.L., Maggese, M.C., Guerrero, G.A., 2009. Exposure to waterborne 4-tert-octylphenol induces vitellogenin synthesis and disrupts testis morphology in the South American freshwater fish *Cichlasoma dimerus* (Teleostei, Perciformes). Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 150, 298–306. doi:10.1016/j.cbpc.2009.05.012

Veado, M.A.R.V., Oliveira, A.H. De, Veado, J.C.C., Revel, G., Pinte, G., 2000. Analysis and distribution of metals in the Paraopeba and the Das Velhas Rivers, Brazil. Water SA 26, 249–254.

Veloso-Júnior, V.C., Guimarães-Cruz, R.J., Barros, M.D.M., Barata, R.S.L., Santos, J.E., 2009. Reproduction of the lambari *Astyanax scabripinnis* (Jenyns, 1842)

(Pisces: Characidae) in a small stream in Southeastern Brazil. J. Appl. Ichthyol. 25, 314–320. doi:10.1111/j.1439-0426.2008.01152.x

Venâncio, L.P.R., Domingos, C.R.., 2014. Effects of Pollutants on Condition Index, in: Almeida, E.A. de, Ribeiro, C.A.O. (Eds.), Pollution and Fish Health in Tropical Ecosystems. Taylor & Francis, London, p. 394.

Viñas, J., Piferrer, F., 2008. Stage-Specific Gene Expression During Fish Spermatogenesis as Determined by Laser-Capture Microdissection and Quantitative-PCR in Sea Bass (*Dicentrarchus labrax*) Gonads1. Biol. Reprod. 79, 738–747. doi:10.1095/biolreprod.108.069708

Wallace, R.A., Selman, K., 1990. Ultrastructural aspects of oogenesis and oocyte growth in fish and amphibians. J. Electron Microsc. Tech. 16, 175–201.

Weigel, N.L., 1996. Steroid hormone receptors and their regulation by phosphorylation. Biochem. J. 319, 657–667.

Wen, R., Xie, Y., Wan, C., Fang, Z., 2013. Estrogenic and androgenic effects in mosquitofish (*Gambusia affinis*) from streams contaminated by municipal effluent in Guangzhou, China. Aquat. Toxicol. 132–133, 165–172. doi:10.1016/j.aquatox.2013.02.010

Yin, P., Li, Y., Chen, Q., Liu, Z., 2017. Diethylstilbestrol , flutamide and their combination impaired the spermatogenesis of male adult zebrafish through disrupting HPG axis , meiosis and apoptosis. Aquat. Toxicol. 185, 129–137. doi:10.1016/j.aquatox.2017.02.013

Zheng, B., Liu, R., Liu, Y., Jin, F., An, L., 2015. Phenolic endocrine-disrupting chemicals and intersex in wild crucian carp from Hun River, China. Chemosphere 120, 743–749. doi:10.1016/j.chemosphere.2014.10.049

Zhou, Q., Li, Y., Nie, R., Friel, P., Mitchell, D., Evanoff, R.M., Pouchnik, D., Banasik, B., McCarrey, J.R., Small, C., Griswold, M.D., 2008. Expression of Stimulated by Retinoic Acid Gene 8 (Stra8) and Maturation of Murine Gonocytes and Spermatogonia Induced by Retinoic Acid In Vitro1. Biol. Reprod. 78, 537–545. doi:10.1095/biolreprod.107.064337